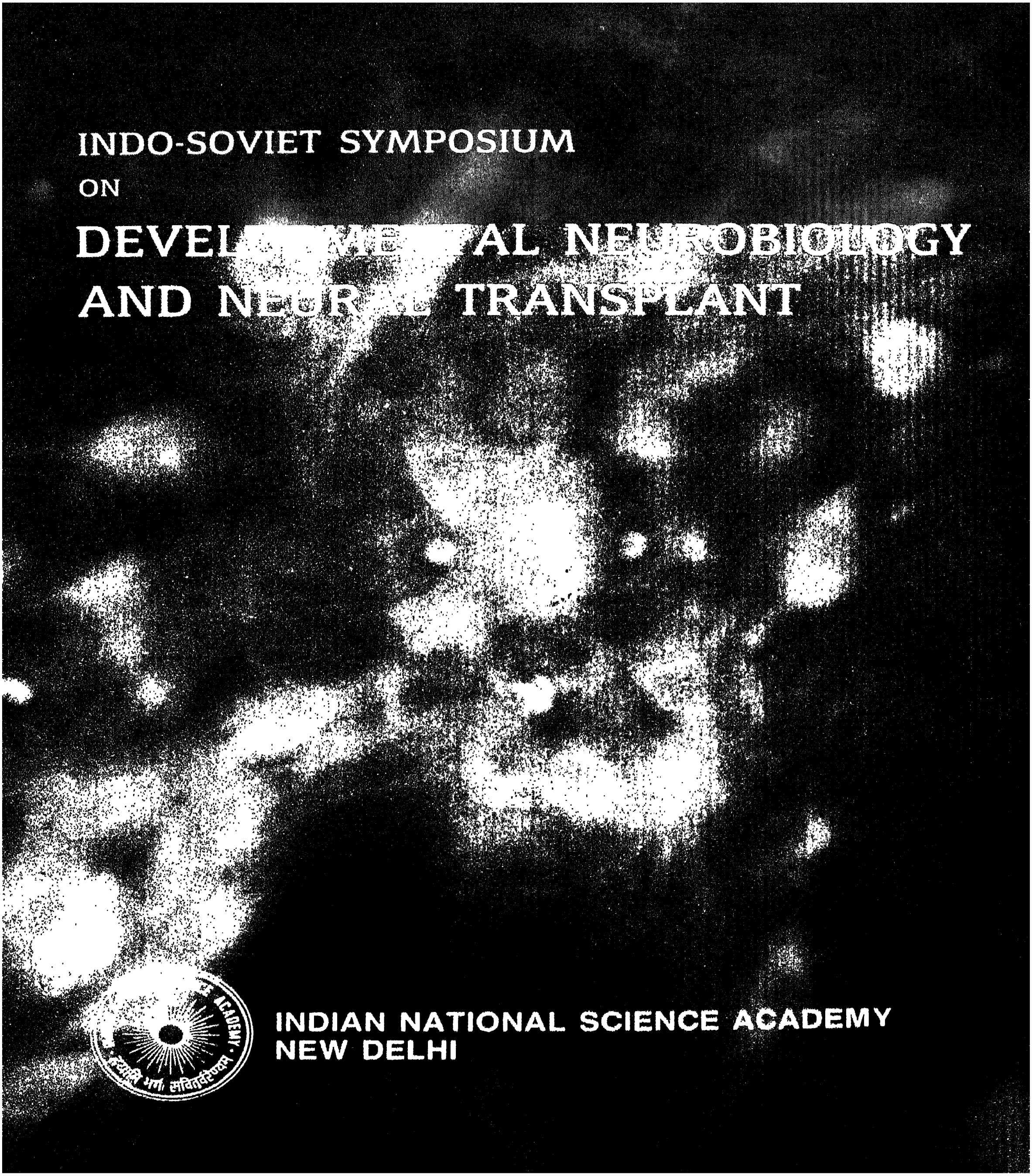


INDO-SOVIET SYMPOSIUM
ON

DEVELOPMENTAL NEUROBIOLOGY AND NEURAL TRANSPLANT

INDIAN NATIONAL SCIENCE ACADEMY
NEW DELHI

INDO-SOVIET SYMPOSIUM
ON
DEVELOPMENTAL NEUROBIOLOGY AND NEURAL TRANSPLANT

held on NOVEMBER 25-28, 1988

Subject Editors: Prof. P N TANDON and Prof. G GOPINATH

Sponsored by
INDIAN NATIONAL SCIENCE ACADEMY
USSR ACADEMY OF SCIENCES

Co-sponsored by
DEPARTMENT OF SCIENCE & TECHNOLOGY, NEW DELHI
COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH, NEW DELHI
INDIAN COUNCIL OF MEDICAL RESEARCH, NEW DELHI
NATIONAL ACADEMY OF MEDICAL SCIENCES, NEW DELHI

Editor of Publications
Prof. V L CHOPRA

Associate Editor
Dr M DHARA

Assistant Editor
JANAK RAJ

Price: Rs. 100 $ 33.00

1990
© INDIAN NATIONAL SCIENCE ACADEMY, NEW DELHI, INDIA

PRINTED IN INDIA

Printed and Published by the Executive Secretary, Indian National Science Academy, Bahadur Shah Zafar Marg, New Delhi-110002 (India) and
Printed by Nirmal Vijay Printers, T-304, Baljeet Nagar, New Delhi-110008. Phone: 5725388, 5728975

CONTENTS

Page

Preface iii

Recommendations iv

Development of Spinal Dorsal Grey Neurons in Man **V Bijlani**, **S Wadhwa** and **T A Rizvi** 1

Neurotransmitter Development in the Human Lateral Geniculate Nucleus **Shashi Wadhwa** and **Veena Bijlani** 7

Spatial Orientation of Cells and Intercellular Interactions in Neuro-ontogenesis **N N Kokina** 17

Effect of Neonatal Undernutrition on the Brain **Chitra Sarkar**, **Subimal Roy** and **Gomathy Gopinath** 29

Effect of Neonatal Undernutrition on Neuromuscular System **Subimal Roy**, **Gomathy Gopinath**, **Madhu G Karmarkar**, **Amit K Dinda** and **Chitra Sarkar** 37

Effect of Malnutrition and Iron Deficiency on Mental Functions and Study of Possible Mechanism in Animal Model **K N Agarwal** 43

Neural Transplantation in Mammals: Our Experiences **P N Tandon**, **Gomathy Gopinath**, **A K Mahapatra** and **A K Shetty** 51

Morphological and Electrophysiological Criteria of the Degree of Integration of the Homo-and Heterotopic Grafts with the Host Brain **A G Bragin** 59

Alterations in Neuronal Development under Influence of Conditions of Nurture and Heterotopic Transplantation **T Desiraju** 69

Intracerebral Transplantation of Cultivated Glio-Neuronal Aggregates and Fetal Brain Tissue Fragments **Ilya V Victorov** and **Anatoly A Lyjin** 73

Transplantation of Embryonic Nervous Tissue Stimulates Compensatory-Restrorative Process in the Brain of Mammals with Diffuse Distrophy of Neurons **M A Alexandrova** 85

MPTP Model of Parkinson's Disease in Monkey: Implications for Neural Transplantation **G Gopinath**, **A K Mahapatra**, **J C Bhardwaj**, **R Banerjee**, **A K Shetty**, **D N Sharma** and **P N Tandan** 95

Neurotransplant Influences upon Amygdalectomized Rats Behaviour **V P Podachin** 101

Biology of Tumour Transplant and Fetal Brain Transplant into Adult Rat Brain **S Mohanty** 107

Perspectives and Limitations of Neurotransplantation **O S Vinogradova** 109

Ultracytochemical Study of the Surface Membrane Components of Nerve Cells Developing in Monolayer Cultures **G G Skibo** and **L M Koval** 125

DNA-Repair in Developing and Aging Brain **Kalluri Subba Rao** 141

Abstracts 151

List of Participants 155

PREFACE

Developmental neurobiology is an emerging discipline of neurosciences. Recent advances in molecular biology, newer morphological and biochemical techniques have now made it possible to get a better and more comprehensive picture of cellular maturation, migration and organisation, not hitherto possible. There are a few individuals/groups in India who are now pursuing these studies. Likewise there are several groups in the Soviet Union actively involved in this kind of research. With this in mind INSA decided to have a joint Symposium between Indian and Soviet Scientists to provide an opportunity for exchange of information and ideas at the highest level.

The first Indo-Soviet Symposium on 'Developmental Neurobiology and Neural Transplant' was held from November 25-28, 1988 in the Indian National Science Academy under the framework of INSA-USSR Academy of Sciences collaborative programme. The Symposium was inaugurated by the then President, INSA, Prof. A S Paintal. Soviet delegation was led by Dr V I Podachin and on Indian side by Prof. P N Tandon. Besides the formal presentation of the scientific data, the symposium provided an opportunity to the scientists from two countries for indepth discussion on Neurosciences. These discussions led to identification of specific areas of common interest for future collaboration.

The volume covers various topics pertaining to Neurobiology, like-Cell Kinetic, Migration and Maturation Factors, Connection and Specificity, Synaptogenesis, Neuronal Plasticity, Neural Transplant, and Nerve Growth Factor and other trophic factors exposing the participants to the current scenario in the field.

RECOMMENDATIONS

Following the scientific deliberations there was detailed discussion on continuing activities for future. It was unanimously agreed that this symposium provided a very useful opportunity not only to discuss the specific scientific subjects but also to familiarise the two sides of the major on-going activities in this field in the two countries. As a sequel of these discussions, both the sides agreed as follows:

1. Second Symposium would be organised in the Soviet Union sometime the in summer of 1990. This Symposium would be on "Plasticity: Development, Regeneration and Transplant". Both sides will then take stock of the advances in Developmental Neurobiology and Neural Transplant and also add new dimension to enhance its scope.
2. It was generally felt by both sides that there should be a mechanism for increasing communication between the scientists of the institutions already identified for collaboration under the joint programme. Each side will provide information with regards to their present and future research interests, scientists involved in various programmes, and publications by the participating institutions.
3. The list of the identified areas in the field of Neurobiology including Higher Nervous Activity as already identified was discussed in detail. It was felt that this provides adequate basis for our continuing interaction. Since, the present Indo-Soviet collaboration programme is operative upto 1990, both the sides should make suggestions to their respective Academies for modification, additions with regards to institutes to be involved, new areas of collaboration or any other item of common interest. The participating institutions from the two sides will make suggestions to their respective coordinators who would interact with each other to make the programme more purposeful. At the moment, two new areas identified were Neuroimmunology: Neurocytology for consideration for future collaboration.
4. It was decided that the proceedings of the symposium will be published by the INSA on the lines of earlier publications brought out by INSA for such a joint programme. Prof. Podachin and Prof. Tandon will collect the manuscripts from the delegates of their respective countries and edit these.
5. Attempts will be made by both sides to provide information regarding scientific activities in the entire field of 'Neurobiology' in the respective countries, over and above the information regarding the participating centres.

It was the unanimous opinion of all the participants that this programme should be extended beyond 1990.

Discussion was also held to activate the exchange programme both at the young and the senior scientists level to strengthen this interaction and ultimately to enable the two sides to identify the areas of collaborative research.

P N TANDON
Indian National Science Academy

V I PODACHIN
USSR Academy of Sciences

INDO-SOVIET SYMPOSIUM
ON
DEVELOPMENTAL NEUROBIOLOGY AND NEURAL TRANSPLANT
NOVEMBER 25-28, 1985
INDIAN NATIONAL SCIENCE ACADEMY

images

images

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 1-6 (1990)

Development of Spinal Dorsal Grey Neurons in Man

V BIJLANI, S WADHWA and T A RIZVI
Department of Anatomy, All India Institute of Medical Sciences, New Delhi 110029

Lamination became apparent at 13-14 weeks and the dorsal grey acquires its adult shape by 30 weeks of intrauterine life. The cells in the deeper laminae were the first to start differentiating followed by those in lamina I and the substantia gelatinosa being the last. The differentiation of laminae, neurons and dendritic arborization appeared to be dependent on the ingrowing afferent trajectory. At 13-14 weeks of gestation projection and interneurons were identifiable in lamina I, primitive-stalked, islet, bipolar cells in substantia gelatinosa and multipolar neurons in the deeper laminae. Dendritic development and remodelling in the interneurons occur red somewhat later than the projection neurons.

Immunohistochemical methods demonstrated presence of substance P at 8 weeks, serotonin at 12 weeks and enkephalin at 14 weeks of gestational age. The immunoreactivity increased with the age. The sequence of synaptogenesis was axodendritic (asymmetrical), axosomatic and axoaxonic in marginal zone, axodendritic (symmetrical), axoaxonica and axosomatic in substantia gelatinosa, axodendritic (asymmetric), axosomatic and serial type in the deeper-zone. There was evidence of the existence of a complex microcircuitry to which the nociceptive input is subjected in the dorsal grey prior to projection to higher centres.

Key Words: Spinal cord, Dorsal horn development, Substance P, Man

Introduction

The dorsal grey of spinal cord has a complex structure and consists of several different neuronal populations. When viewed in transverse section, the cellular columns appear as layers. Rexed (1952) described these as laminae—laminae I to VI are found in the dorsal grey. Lamina I is a thin layer of grey matter and is also called marginal zone. Laminae II and III, according to most descriptions correspond to the region of substantia gelatinosa. Most of the spinothalamic fibres are described to originate lamina V, some also arise from laminae I, VII and VIII.

The present study reports the development of spinal dorsal grey in human foetuses from early period of gestation (when the neuropil is simpler) through the later periods (when the connectivity develops). It may be helpful in understanding the various components of neural circuits which take part in processing of incoming sensations.

Materials and Methods

The investigation was carried out on human foetal material obtained from hysterotomy performed for medical termination of pregnancy with the prior consent of the parents. The foetuses ranged in age from 8 to 37 weeks of gestation. The spinal cord from 4th to 8th cervical segments was dissected. The dorsal half was isolated and processed for light microscopy, electron microscopy and immunocytochemical investigation for localising substance P, enkephalin and serotonin.

Development of the neuronal somata was studied on Nissl stained material, growth of dendritic spread was studied by golgi stain, and avidin biotin complex modification of Sternberger's technique (1974) was used for localisation of substance P, enkephalin and serotonin, using monoclonal antibodies. Observations were made on the sequence of development of various types of synaptic profiles in the different laminae.

Results

First appearance of lamination started at 13-14 weeks. With increase in age the laminae became more distinct. The dorsal horn acquired adult shape by 30 weeks (figure 1). At this age laminae I to VI could be distinguished. At 13-14 weeks, marginal zone (laminae I), substantia gelatinosa (laminae II & III) and deeper zone neurons could be distinguished clearly by Nissl stain—the neurons in deeper zone appeared to be more mature compared to the marginal zone while the substantia gelatinosa was less mature than both.

There was a progressive decrease in numerical density of the neurons in the marginal zone, substantia gelatinosa and deeper zone between 14 and 26 weeks of gestation. It is interesting to note that at 26 weeks, numerical density in substantia gelatinosa was almost 1/6th of that at 14 weeks and the substantia gelatinosa at this stage appeared like a sheet of cells (figure 2).

With golgi stain, on the basis of soma size, dendritic configuration and axonal course, projection and interneurons were identified in marginal zone. The projection neurons showed characteristic mediolateral spread, dendrites elongated till 25-26 weeks, at which age considerable amount of dendritic remodelling was seen. "Encased neuron"—an interneuron, described in great detail by Lima and Coimbra (1983)—was studied in detail. Dendritic remodelling of this neuron occurred at somewhat later date (figure 3). In the substantia gelatinosa several types of interneurons—bipolar and multipolar, with dorsal dendritic spread (islet), with ventral dendritic spread and dorsally directed axon (stalked)—were identified. On the whole, the substantia gelatinosa neurons appeared to have less mature characteristics at all age periods as compared to marginal and deeper zones. Golgi stain also illustrated marginal plexus of fibres extending along the margins of the grey matter at 13-14 weeks of age. At 23-24

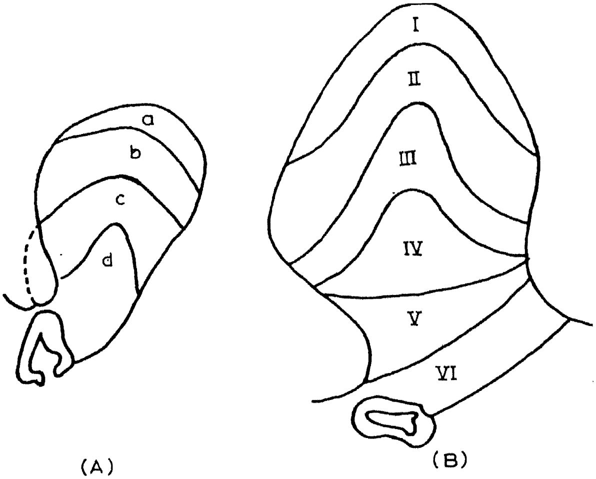


Figure 1 Schematic diagrams of dorsal horn of human fetal spinal cord at 13-14 (**A**) and 30 weeks (**B**), showing formation of Rexed's laminae

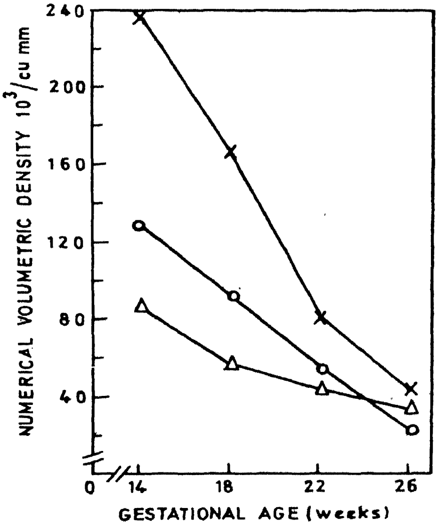
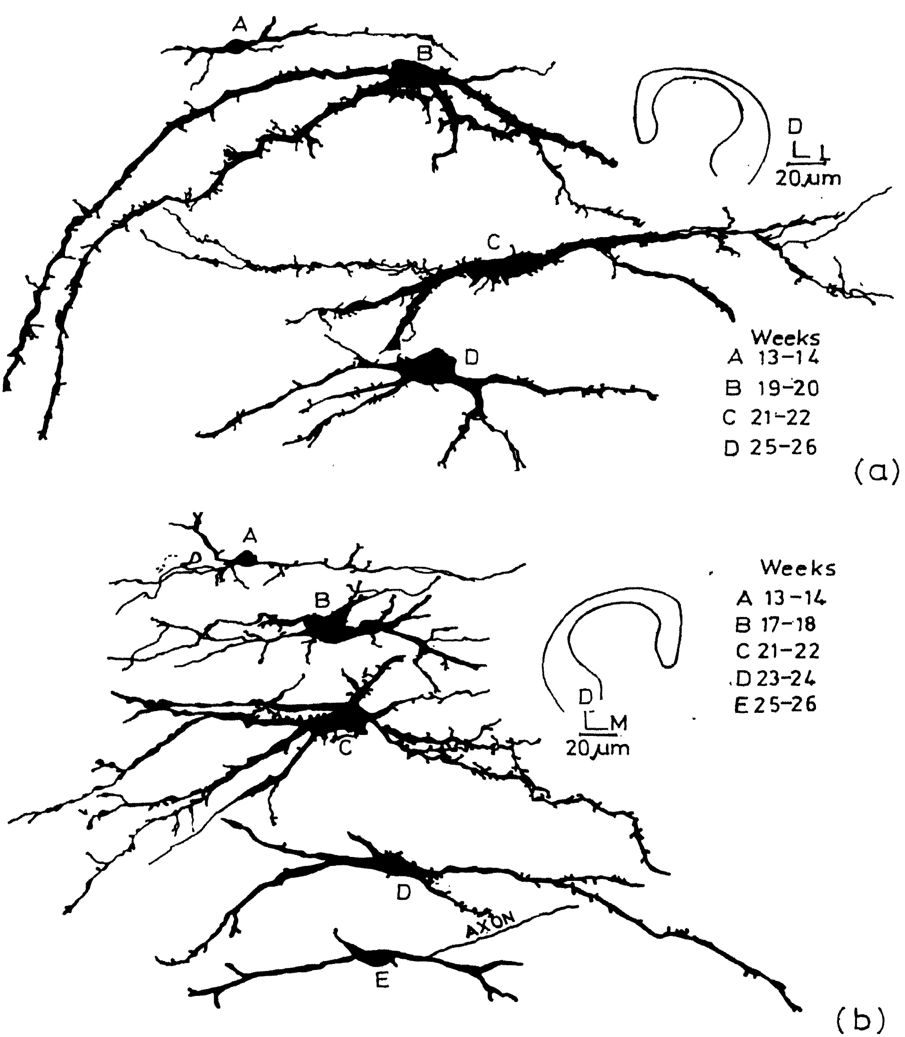


Figure 2 Graph shows a fall in numerical density from 14-26 weeks of gestation in all the three zones. The fall is maximum in substantia gelatinosa region. Δ—Δ Marginal zone, ×—× Substantia gelatinosa 0—0 Deeper zone



3 Camera lucida drawings of Golgi stained neurons, showing dendritic changes during development of projection interneurons (B) at different age periods mentioned on the right side weeks, these fibres were seen curving in dorsally into the middle of the dorsal horn forming a prominent dense plexus in laminae II and III. The growth of fibres as observed in golgi preparations appeared to be influencing the pattern of dendritic arborization and maturation (figure 4).

Substance P positivity identified nociceptive primary afferent fibres. This activity was seen in the marginal zone from 8 weeks onwards. The density of reactivity increased with the age—the maximum being at 37 weeks of age.

Enkephalin immunoreactivity appeared later at 14 weeks of age, it also increased with the age and the pattern of staining was similar to substance P. Serotonin activity appeared at 12 weeks. The pattern was somewhat different than substance P and enkephalin. The activity was in the form of punctated honey comb dots.

The sequence of synaptogenesis was axodendritic (asymmetrical), axosomatic and axoaxonic in marginal zone, axodendritic (symmetrical), axoaxonic and axosomatic in substantia gelatinosa, axodendritic (asymmetrical), axosomatic and serial type in the deeper zone.

Discussion

In recent past, the dorsal grey of spinal cord has been the subject of intensive investigations. Gobel (1978a, b) using golgi studies has reported a variety of neurons in the lamina I. He classified these into smooth pyramidal, spiny pyramidal, loose multipolar and compact multipolar types. According to him, all are projection neurons. He denied the presence of interneurons in the marginal zone. Beal et al. (1981) classified these neurons into groups I to IV. Lima and Coimbra (1983) using silver stained semithin serial sections of lamina I identified four types of neurons, namely fusiform, flattened, multipolar and prismatic. Flattened cells observed by him are projection neurons and correspond to the Waldeyer's cells. The other three types are interneurons.

In the present study large neurons with a mediolateral dendritic spread are seen situated along the dorsal margin. From their axonal course and size of the soma these appear to be projection neurons and correspond to the flattened cells of Lima and Coimbra (1983). These neurons, at the age of 13-14 weeks, have small dendrites with few spines and smooth soma. With advancing age the dendrites grow longer, branching increases and the number of spines also increases. At 17-18 weeks, spines are seen on the soma. At 22 weeks, the soma has many more spines and the secondary and tertiary branching of dendrites is seen. After this age between 25 and 26 weeks considerable remodelling occurs (figure 3A). Figure 3A also shows medially directed dendrites of the projection neurons at different age periods. The features described above are observed in all developing neurons. The role of somatic spines is not clear. These could be the sites of cytoplasm later to be drawn into the processes, or the sites of contacts with ingrowing primary afferent fibres. Coss and Perkel (1985) have elaborately described the role of spines in electrical activity. These have special property of enhancing the transfer of electrical changes on stimulation. Axosomatic synapses were seen at 17-18 weeks. In golgi preparations sometimes an apparent contact with primary afferent fibre is visible. If the activity proposed by Coss and Perkel (1985) is transferred onto the spines seen in a developing neuron, then it appears that the spines help in transfer of electrical activity from the developmental afferent trajectory; and once the neuron reaches a certain stage of development, then this role of the spines probably ceases to exist. Spine elimination appears to be a regressive event. There is not enough information on this event in literature. We have not encountered any prominent cell death. One wonders whether cell death is dominant in this region in foetal life or not. While not denying its existence (for lack of concrete proof), the evidence is more towards the dendritic and spine elimination which could be a more prominent mechanism for loosing redundancy and chiselling pathways. Development of interneurons is little later than the projection neurons (figure 3B). Similar features of spiny stage and later dendritic remodelling are seen.

At 13-14 weeks, with Nissl staining, substantia gelatinosa or laminae II and III are seen consisting of dense cords of cells. The density of this region decreases steeply with the age (figure 2). This probably is due to the ingrowing afferent trajectory. The primary afferent fibres are seen in golgi stained material as dense marginal plexuses. At 13-14 weeks these are seen extending along lateral and medial margins, curving in

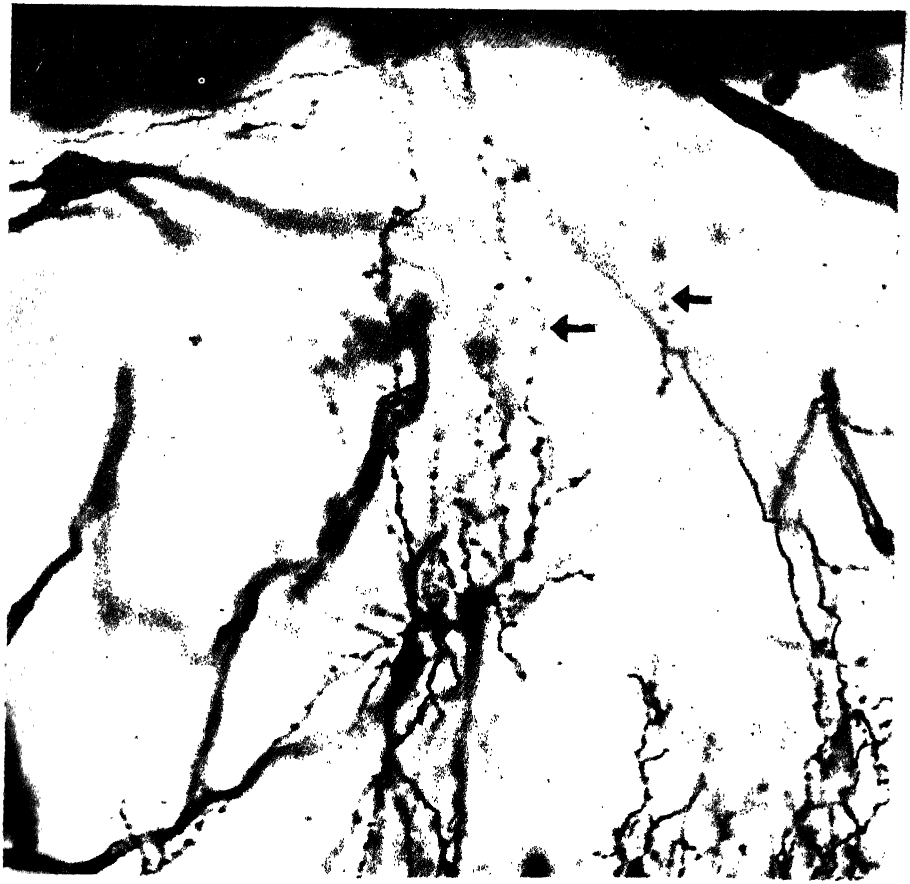


Figure 4 Substantia gelatinosa neurons showing dorsally directed beaded dendrites. Arrows indicate primary afferent fibres coursing towards the developing dendrites

towards lamina III at 23-24 weeks. These afferents invade and form dense arbors in the region of substantia gelatinosa. Some fibres are seen coursing dorsoventrally from the marginal zone. Immunocytochemically these fibres are substance P positive. Punctate appearance of serotonin fibres indicates (these to be) cut ends. These are probably of supraspinal origin. They form dense plexuses around the neurons. The growth of all these fibres contributes to the sharp fall in the numerical density in the region of substantia gelatinosa (figure 2).

Several varieties of substantia gelatinosa neurons have been mentioned in the literature. Gobel (1975, 1978a,b) has described islet cells, stalked cells, spiny cells, arboreal cells and border cells. Schoenen (1982) has classified the same neurons as islet cells, filamentous cells, curly cells and stellate cells. Axons of islet cells remain in the vicinity in the same lamina. Gobel considered these to be inhibitory neurons. Others have described nociceptive islet cells. Bennett et al. (1979) stimulated 12 stalked cells in lumbosacral region of cat. According to him six were found to be wide dynamic range neurons and other six were excited by noxious stimuli.

Substantia gelatinosa is a region of the spinal cord where the afferent fibres enter from the dorsal side directly from the spinal roots. The thick bundles of fibres run along the medial and lateral border of the grey matter. These fibres also give collaterals all along the edges of the grey matter. Figure 4 shows one substantia gelatinosa neuron with beaded dendrites directed dorsally. These dorsal dendrites are immature as compared to the ventral dendrites which are approached by afferent fibres earlier. The dendrites of developing neurons seem to grow towards the afferent fibres which provide the sensory input. This results in a variety of neuronal shapes (Bijlani 1988).

In an excellent review, Wall (1988) has criticised the concept of hard wired dedicated modality specific system in spinal dorsal grey. According to him, there is plasticity in adult nervous system and the processing of sensory information is in context with other inputs from periphery and centre. Thus, advocating no fixed relationship between the input and output of individual dorsal grey neurons. Our studies support this concept. Neuronal shapes are dependant on the templating forces in the environment, which provide guidance and constraining mechanisms to the cytological machinery of the cell.

Sequence of synaptogenesis is similar in marginal zone and deeper zone. Both these regions have prominent projection neurons. The sequence is different in the substantia gelatinosa which is densely populated with interneurons. Interneurons provide versatility of activity in a neuronal assembly. In substantia gelatinosa, enkephalin, and substance P immunopositivity is seen. Terminals of supraspinal serotoninergic fibres form rich plexuses around these neurons. Ruda and Gobel (1980) have described the probable mechanism of action of these neurons which may be influenced in multiple ways by the rich biochemical variety of fibres. These interneurons influence the activity of the projection neurons and play an important role in processing the sensory input to the dorsal grey.

In conclusion, in the dorsal spinal grey of human foetuses, the projection neurons develop earlier than the interneurons. Neuronal shapes and dendritic arborization appear to depend on primary afferent trajectory. Dendritic and spine elimination is a prominent regressive feature during development. Substantia gelatinosa, a region overpopulated with interneurons, shows dominance of inhibitory type of synapses in comparison to other parts of the dorsal grey.

References

Beal J A, Penny J E and Bicknell H R 1981 Structural diversity of marginal (lamina I) neurons in the adult monkey (*Macaca mulatta*) lumbosacral spinal cord. A Golgi study; *J. Comp. Neurol.* **202** 237-254

Bennett G J, Hayashi H, Abdelmoumene M and Dubner R 1979 Physiological properties of stalked cells of the substantia gelatinosa intracellularly stained horseradish peroxidase; *Brain Res.* **164** 285-289

Bijlani V 1988 Cellular basis of neurobiology; in *Lectures in Neurobiology* eds P N Tandon, V Bijlani and S Wadhwa, (Delhi: Rakino Press Pvt. Ltd. for Everyman's)

Coss R G and Perkel D H 1985 The function of dendritic spines. A review of theoretical issues; *Behavi. Neural Bio.* **44** 151-185

Gobel S 1975 Golgi studies of the substantia gelatinosa neurons in the spinal trigeminal nucleus; *J. Comp. Neurol.* **162** 397-415

—— 1978a Golgi studies of the neurons in layer I of the dorsal horn of the medulla (trigeminal nucleus caudalis); *J. Comp. Neurol.* **180** 375-394

—— 1978b Golgi studies of the neurons in layer II of dorsal horn of the medulla (trigeminal nucleus caudalis); *J. Comp. Neurol.* **180** 395-414

Lima D and Coimbra A 1983 The neuronal population of the marginal zone (lamina I of the rat spinal cord). A study based on reconstructions of serially sectioned cells; *Anat. Embryol.* **167** 273-288

Rexed B 1952 The cytoarchitectonic organization of the spinal cord in the cat; *J. Comp. Neurol.* **96** 415-495

Ruda M A and Gobel S 1980 Ultrastructural characterization of axonal endings in the substantia gelatinosa which take up serotonin; *Brain Res.* **184** 57-83

Schoenen J 1982 The dendritic organization of the human spinal cord. The dorsal horn; *J. Neurosci.* **7** 2057-2081

Sternberger L A 1974 *Immunocytochemistry* (Englewood Cliffs NJ: Prentice Hall, Inc.) 246 pp

Wall P D 1988 Stability and instability of central pain mechanism (Chapter 2); in *Pain Research and Clinical Management*, pp 14-24 eds R Dubner, G F Gebhart and M R Bond; *Proc. 5th World Congress on Pain.*

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 7-16 (1990)

Neurotransmitter Development in the Human Lateral Geniculate Nucleus

SHASHI WADHWA and VEENA BIJLANI
Department of Anatomy, All India Institute of Medical Science, New Delhi 110029 (India)

Histochemical and immunohistochemical studies were conducted on the lateral geniculate nucleus obtained from human fetuses and premature neonates ranging in age from 8 to 37 weeks of gestation to determine the developmental profile of the acetyecholinesterase, catecholamine, gamma-aminobutyric acid (GABA), substance P and serotonin. It was observed that the different neurotransmitters/neuromodulators showed different developmental patterns. The catecholamine fibres increased with advancing gestational age, while substance P and serotonin fibres increased upto 16-17 weeks of gestation and thereafter declined. Acetylcholinesterase activity appeared at 17-18 weeks of gestation and concentrated in the interlaminar zones at 26 weeks. GABA ergic cells appear at 15-16 weeks of gestation, attained maximum density at 17 weeks and stared declining by 19 weeks. At 26 weeks only 5% cells were GABA-immunopositive. The significance and probable role of the developmental changes in the transmitter profiles are discussed.

Key Words: Neurotransmitters, Development, Lateral geniculate nucleus (LGN), Man, Prenatal life

Introduction

The lateral geniculate nucleus (LGN) is the first site of interaction between optic nerve afferents from both the eyes, resident population of neurons and additional afferents from the cortex, reticular formation, locus coeruleus, dorsal raphe nuclei, perigeniculate nucleus and superior colliculi. Some details of the neurochemical circuitry in this nucleus have been analysed in the adult mammals. Development of neurotransmitter profile of glutamate, γ-aminobutyric acid (GABA) and choline-acetyltransferase has been studied in the lateral geniculate nucleus (LGN), superior colliculi and visual cortex of postnatal albino rats (Kvale et al. 1983). GABA uptake in rat LGN (Coyle & Enna 1976) and biochemical estimation of glutamic acid decarboxylase (GAD), an enzyme for synthesis of GABA show increasing activity in postnatal life (McDonald et al. 1981).

Studies on the presence of AChE during development indicate existence of a transient pattern in the visual cortex of rat (Robertson et al. 1985), prefrontal cortex and dorsomedial nucleus of thalamus of monkey (Kostovic & Rakic 1984). Regenerating optic nerves of anurans (Rana pipiens) have been demonstrated to contain various peptides like substance P, cholecystokinin, L-enkephalin and bombesin (Kuljis et al. 1984) while there is absence of these in the adult. Serotonin (5HT) containing dorsal raphe neurons as well as noradrenergic afferents from locus coeruleus are found to innervate (Pasquier & Villar 1982, Wilson & Hendrikson 1985, Rogawski & Aghajanian 1982) and influence the transmission properties of relay cells of visual thalamus in various states of sleep and wakefulness (Hubel 1960).

Though there is increasing evidence of the appearance and disappearance of immunochemically labelled neurons as well as of afferent and efferent projections in the visual cortex (Parnavelas & Cavanagh 1988), such information in relation to the developing human lateral geniculate nucleus is still not available. Hence the present study was undertaken to investigate the neurotransmitter profiles in the developing human lateral geniculate nucleus during prenatal life.

Material and Methods

Tissues from human fetuses and premature neonates of gestational ages 8 to 37 weeks were obtained from patients undergoing medical termination of pregnancy by hysterotomy at the All India Institute of Medical Sciences and Safderjang Hospital with prior consent of mother and permission of the Ethics Committee.

Semithin sections of LGN's from fetuses of 8,12, 15-16, 17,19,21,22-23, 26 and 37 weeks gestation were processed for immunocytochemical staining of GABA neurons with Sternberger's peroxidase anti-peroxidase method using polyclonal antibodies.

Substance P and serotonin were localised on Zamboni fixed cryostat sections (24-32 μm) from fetal and premature brain specimens of ages 12 to 37 weeks of gestation with Avidin Biotin peroxidase technique using monoclonal antibodies.

Fresh frozen cryostat sections from specimens of gestational ages 12,14-15, 17-18 and 26 weeks were stained for catecholamines using the glyoxylic acid method of de la Torre and Surgeon (1976) and LGN's from 13-14, 17-18 and 26 weeks were processed by El Badawi and Schenk's modification of Karnovsky and Roots method (1976) to demonstrate specifically the enzyme acetylcholinesterase (AChE).

Results

The mean density estimates of GABA immunopositive and GABA immunonegative cells at different gestational ages and their relative percentages were made. GABA immunopositive cells constituted 1% of the total population density of cells at 15-16 weeks. At 17 weeks, however, there was a steep rise in the numerical density of GABA immunopositive cells which form 16% of the total neuronal density while there was a fall in the density of GABA negative neurons. At 19 weeks the percentage of GABA immunopositive neurons fell to 4% and remained almost constant at 21 and 22-23 weeks although the numerical density of both GABA immunopositive and GABA immunonegative cells continued to decline. At 26 weeks, while the numerical density of GABA immunopositive neurons declined, their percentage rose to 5%. Estimate of mean density and percentage of GABA positive and negative cells are given in table 1. For detailed observation see Wadhwa et al. (1988b).

Substance P: At 13-14 weeks, a few substance P immunoreactive fibres were seen reaching the LGN through the optic tract. The number of these fibres increased by 16-17 weeks (figure 1 a-e). At 26 and 37 weeks of gestation, occasional substance P positive

Table 1 *Comparison of numerical densities from GABA-stained sections and precentage of GABA-positive and GABA-negative neurons*

Gestational age (weeks)	Nv GABA positive $(\times 10^3/mm)^3 \pm SD$	% GABA positive	Nv GABA negative $(\times 10^3/mm)^3 \pm SD$	% GABA negative
8	—	—	—	—
12	—	—	—	—
15-16	2.03 ± 0.13	0.9	233.53 ± 0.80	99.1
17	18.04 ± 3.38	16.1	94.04 ± 16.30	83.9
19	3.79 ± 0.61	4.6	78.12 ± 6.66	95.4
21	3.23 ± 0.83	4.2	74.44 ± 6.74	95.8
22-23	2.08 ± 0.60	4.3	46.19 ± 2.91	95.7
26	1.77 ± 0.64	5.2	32.60 ± 5.40	94.8

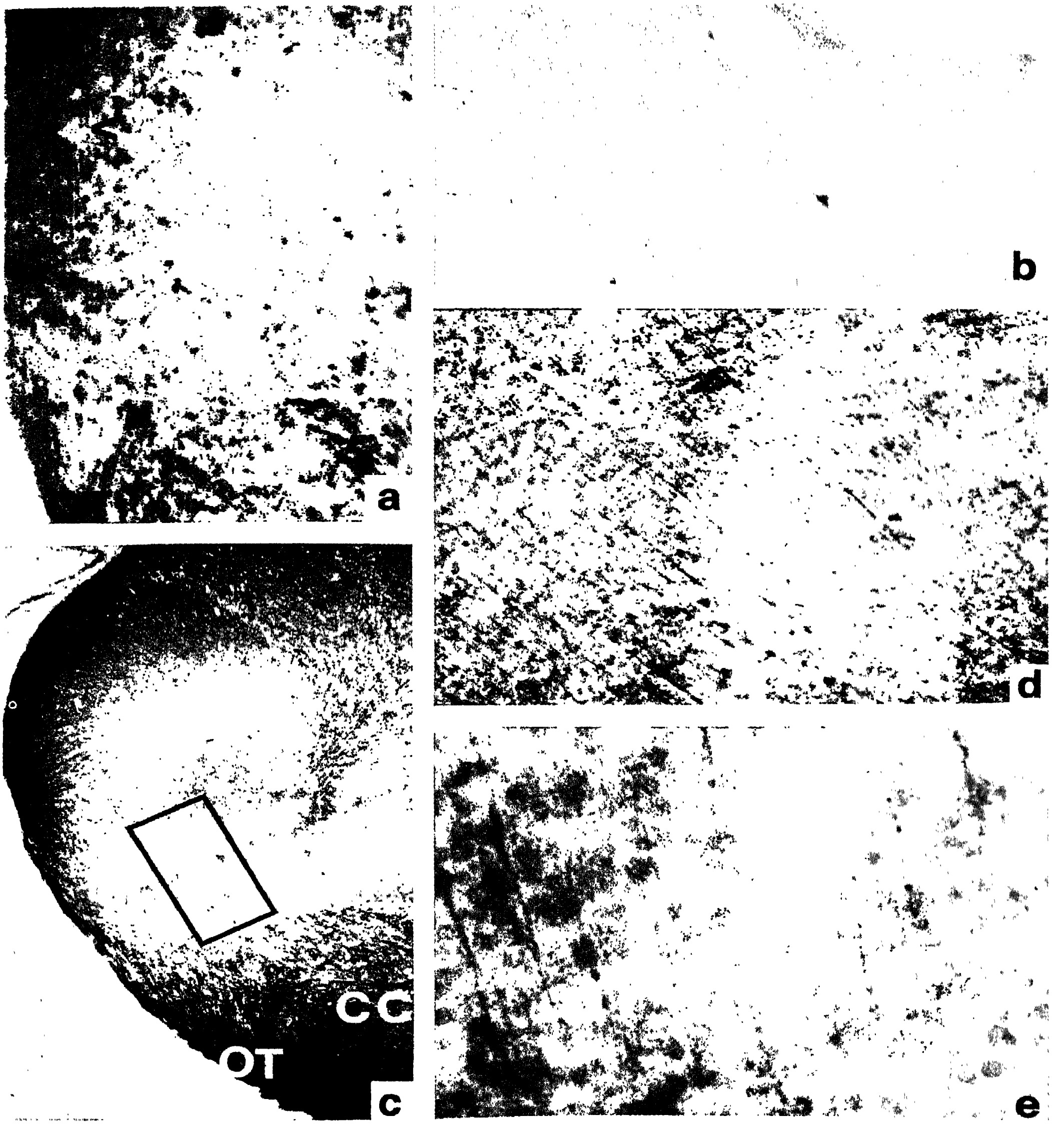


Figure 1a – e, Substance P immunoreactivity in LGN **a,** Photomicrograph of 13-14 weeks LGN shows few immunoreactive fibres **OT**, optic tract X 80; **b,** High power photomicrograph of an individual fibre shown in figure 1a. Note the beaded nature of the SP immunoreactive fibre (× 800); **c,** 16-17 weeks LGN showing larger number of immunoreactive fibres entering through the optic tract (**OT**) (**CC**, crus cerebri) (× 25); **d,** Area outlined in figure 1c viewed at a higher magnification. Note the black varicose fibres (× 370); **e,** The same as in figure 1d viewed at a further higher magnification (× 925)

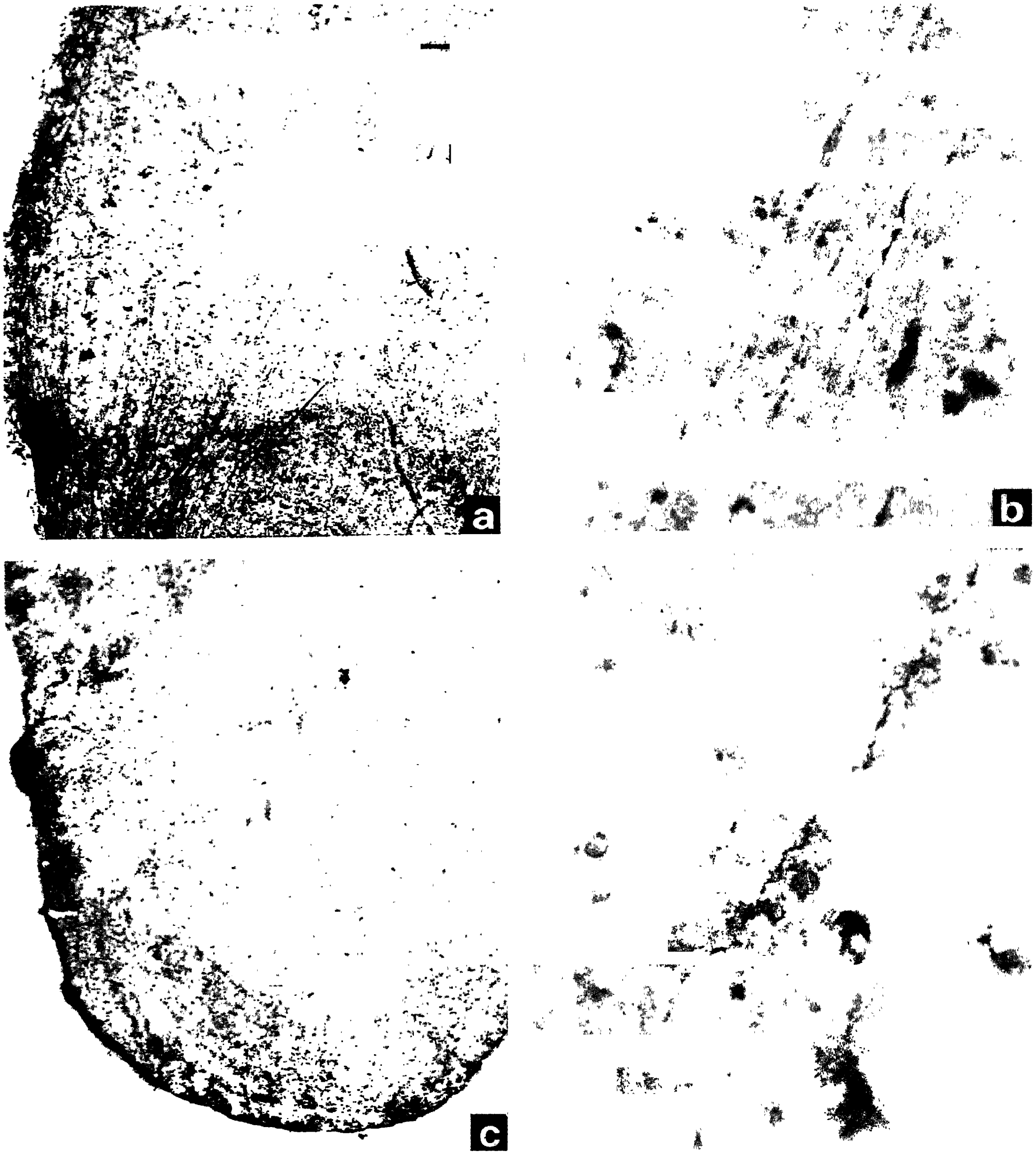


Figure 2a – d, Serotonin immunoreactivity in LGN **a,** 13-14 weeks LGN shows the outline of LGN with the stained fibres (× 92); **b,** Area outlined in figure 2a viewed at a higher magnification shows the direction of these fibres and the adjacent unstained fibres (× 925); **c,** 26 weeks LGN showing its outline (× 40); **d,** Varicose fibres immunopositive for serotonin at 26 weeks seen at a higher magnification (× 925)

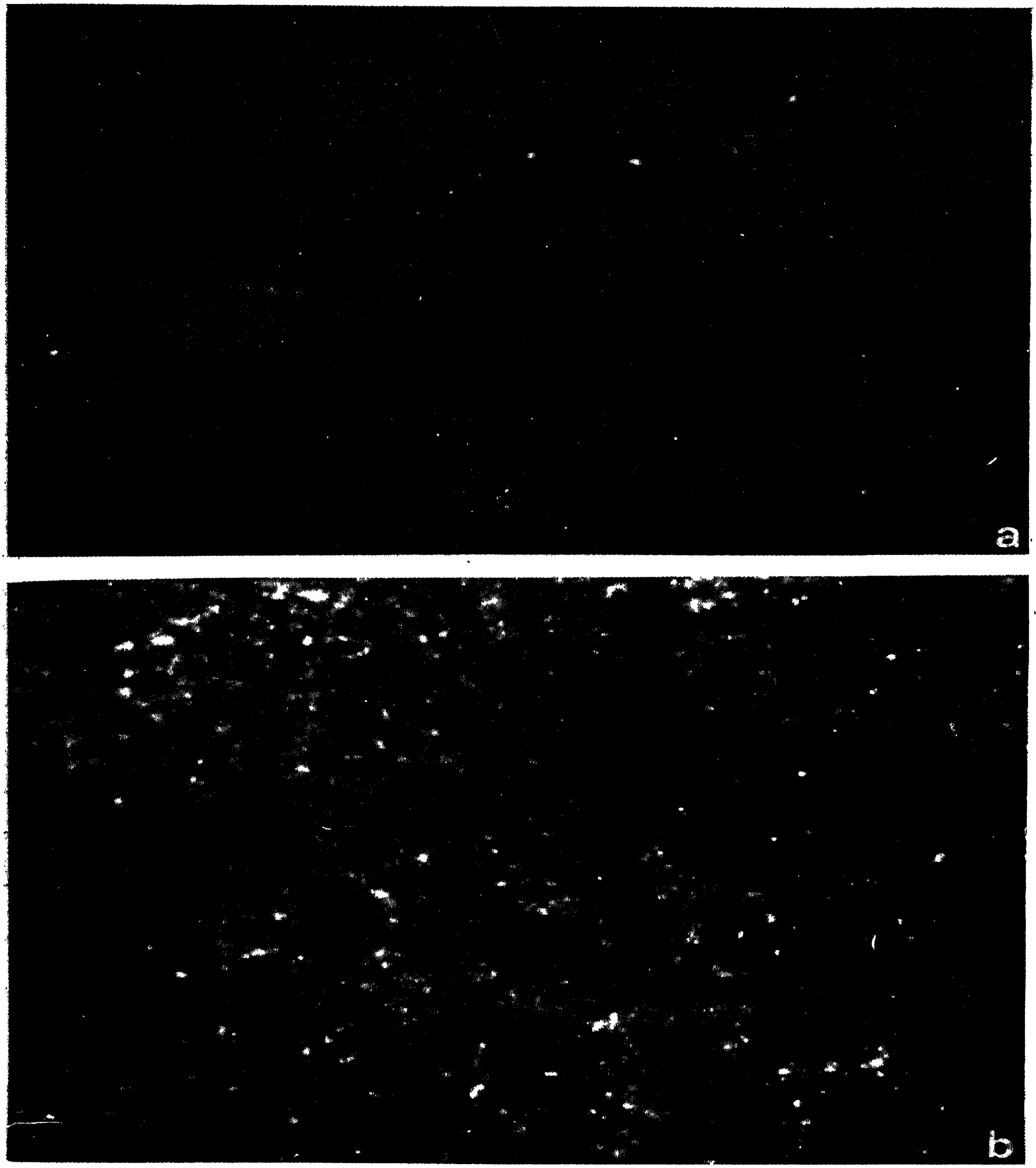


Figure 3a – b, Fluorescene photomicrographs showing adrenergic fibres **a**, 17-18 weeks LGN stained with

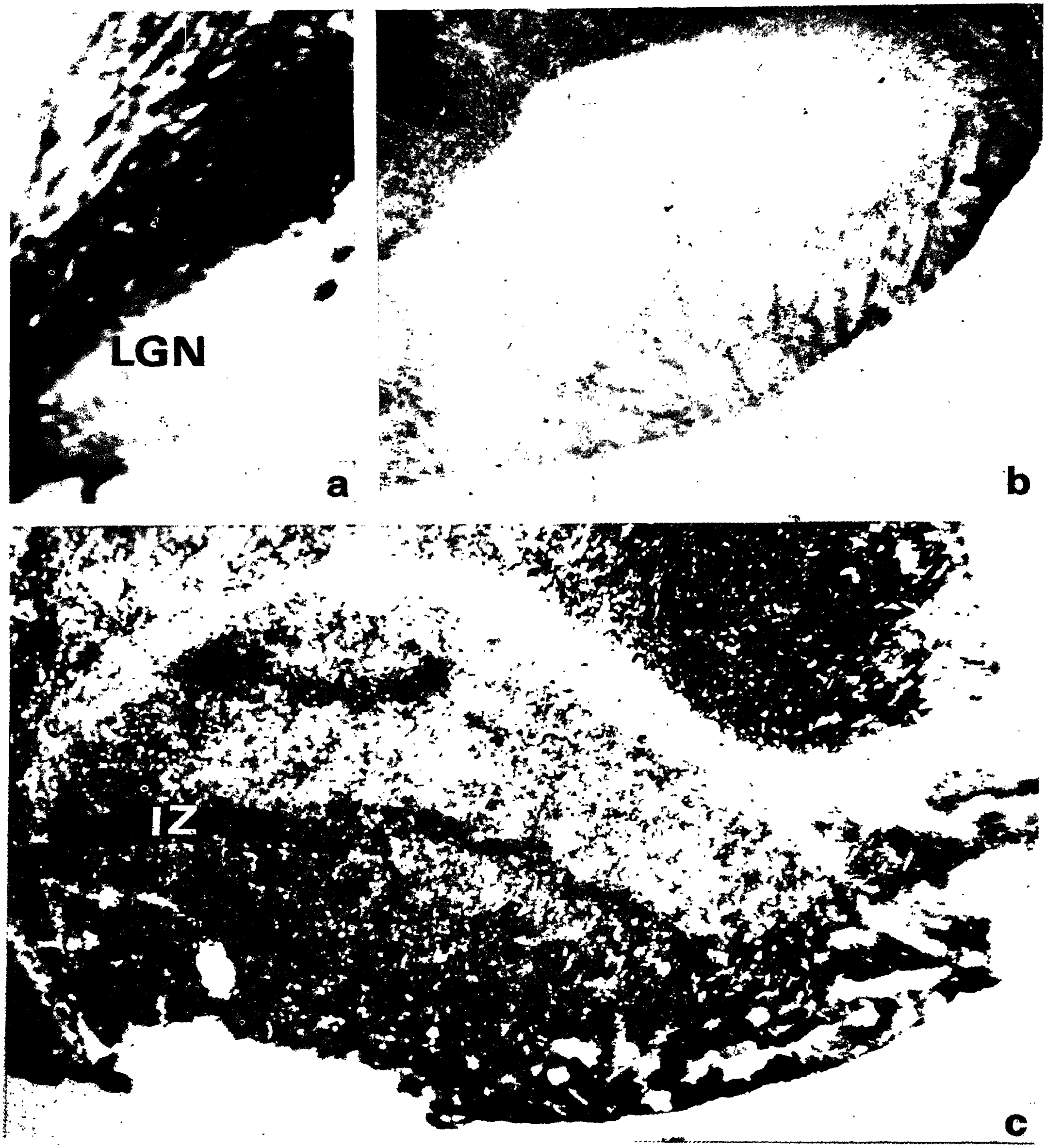


Figure 4a – c, Acetylcholinesterase activity in LGN **a**, 13-14 weeks diencephalon showing the absence of enzyme activity in the LGN (× 25); **b**, 17-18 weeks LGN showing weak activity in its superficial zone (× 25); **c**, 26 weeks LGN showing concentrated activity in the interlaminar zones (IZ) and in the magnocellular layers (× 25)

fibres were found running perpendicularly across the laminae along the lines of retinal fibre projections in the LGN. Detailed observations have been reported earlier (Wadhwa et al. 1988a).

Serotonin: Serotoninergic fibres were observed in 12, 13-14, 16-17, and 26 weeks LGN (figure 2 a-d). Greater number of immunopositive fibres for serotonin were present at 13-14 weeks than at 26 weeks. During the early gestation period fibres were seen entering the LGN from the optic tract.

Catecholamines: Noradrenergic fibres, demonstrated by glyoxylic acid method, were observed in 17-18 and 26 weeks LGN (figure 3 a-b). They appeared as bluish green plexuses of fine varicose fibres. The density of fluorescent varicosities increased with age.

Acetylcholinesterase (AChE): AChE enzyme activity which was not observed at 13-14 weeks (figure 4a), made its appearence at 17-18 weeks in the superficial region of LGN (figure 4b). At 26 weeks the AChE staining was concerntrated in the interlaminar zones while the magnocellular layers showed moderate intensity of staining (figure 4c).

Discussion

The present study has analysed the developmental changes in the acetylcholinesterase, catecholamine, GABA, substance P and serotonin profiles of human lateral geniculate nucleus. It is observed that the different neurotransmitters/neuromodulators show different temporal patterns in development as summarized in table 2.

GABA is perhaps the most satisfactorily documented neurotransmitter in relation to the interneurons of the LGN (Fitzpatrick et al. 1984, Montero & Zempel 1985 Gabbott et al. 1986) and is reported to have an inhibitory role in modulating the receptive field properties of projection neurons (Sillito & Kemp 1983) and orientation selectivity (Vidyasagar 1984). Changes seen in the numerical density and percentage profiles of the GABA neurons in the present study could indicate a phase in the development kinetics of eliminating the overproduced interneurons to reach the adult values. The maximum numbers of GABA immunopositive cells at 16-17 weeks of gestation may have important implications in the development of neural circuity. Evidence is accumulating to suggest that cells transiently present during fetal and postnatal life could influence the pattern of connectivity (Chun et al. 1987). In their studies on the superior cervical ganglia of adult rat, Dames et al. (1985) and Toldi et al. (1986) have demonstrated the influence of GABA and NaBr (known to have inhibitory action on synaptic transmission) in the promotion of synaptogenesis.

Since 1954, there have been reports demonstrating biological activity of substance P in the optic nerve extracts of adult pigeon, rat and cow (Duner et al. 1954, Winder & Patsalos 1974, Reubi & Jessel 1978). These observations are treated cautiously in view of the con-

Table 2 *Some neurotransmitter profiles at different gestational ages*

Transmitter	12 weeks	13-14 weeks	15 weeks	15-16 weeks	16-17 weeks	17-18 weeks	21 weeks	26 weeks	37 weeks
Substance P fibres	+ +				+ + +			+	+
Serotonin fibres	+ +	+ +			+ + +			+	
Noradrenergic fibres	+					+ +		+ + +	
Acetylcholinesterase fibres		—				+	+ + + +		
Gamma aminobutyric acid-terminals	—		+ +	+ +	+ + +		+ + +	+ + +	
Cells	—		—	+	+ + + +		+ +	+ +	

Poor; Slight; Moderate; High; — Not detected tamination with other nerves around optic nerve and in the ciliary ganglion. However, demonstration of substance P like immunoreactivity in some ganglion cells of adult monkey retina (Brecha et al. 1982) and adult rabbit (Brecha et al. 1986) provides evidence for substance P being a neurotransmitter in the retinogeniculate pathway. In the present study, a greater number of substance P immunopositive fibres entering the LGN from the optic tact and also passing beyond it perhaps to the superior colliculi were observed at early gestational periods than at later ages. It is probable that the peptidergic transmission representing perhaps the W cell system is evident more in early gestational ages indicating a repetition of the evolutionary step in ontogeny, with some trophic effects in the developing neural circuitry of LGN.

Acetylcholinesterase has been shown to be transiently present in the developing visual cortex of rat (Robertson et al. 1985). While the presence of AChE in developing tissue has been suggested to play a specific role in the development and differentiation of neural tissue (Krnjevic & Silver 1966), the functional role of the transient activity is not clear. Robertson et al. (1985) in their study on the patterns of AChE in developing rat visual cortex suggested that the AChE activity is characteristic of geniculocortical axon terminals at the stage of establishing functional connections with the postsynaptic sites in the cortex. The geniculocortical source of AChE has been confirmed by them in their recent study (Robertson et al. 1988). In the present study, AChE activity in the LGN appears around 17-18 weeks and gets concentrated in the interlaminar regions at 26 weeks of gestation. A similar pattern has been noted in the rhesus monkey and human LGN by Kostovic and Rakic in 1984. The authors have also demonstrated the subsequent loss of reactivity in the interlaminar spaces with moderate staining in cellular laminae in a 3 months old infant. However, they have not discussed the significance of these observations. Fitzpatrick and Diamond (1980) have pointed out an association of AChE enzyme with corticogeniculate terminals. In the light of this data and our electronmicroscopic observations which indicate the formation of corticogeniculate synapses around 18-19 weeks of gestation (Wadhwa & Bijlani 1988), it is probable that the appearance of AChE activity and subsequent intensity at 26 weeks in the interlaminar zones may be connected with the initial establishment of synaptic connections of corticogeniculate fibres. Subsequent disappearance of the AChE activity from the interlaminar spaces as observed by Kostovic and Rakic (1984) also suggests a role of AChE in the formation of synaptic contacts of the corticogeniculate fibres. Bear et al. (1984) have suggested that in the postnatal period in the kitten striate cortex, acetylcholine may have a stabilishing effect on the marginally effective (transient) synapses which would otherwise be eliminated.

In the present study two aminergic transmitters-noradrenaline and serotonin-were examined. Noradrenaline in the adult LGN is seen to facilitate the excitability of relay neurons, (Nakai & Takaori 1974, Kayama et al. 1982). Noradrenergic projection to the LGN observed from an early gestation period, gradually increased in later periods. It is probable that during these early stages it influences growth mechanism. Parnavelas and Blue (1982) found that neonatal depletion of cortical noradrenaline with 6-hydroxydopamine actually accelerates the rate of synapse formation in developing rat neocortex thereby suggesting that under normal conditions its presence would inhibit synaptogenesis. However, in view of the diverse experimental data available in the recent years, reviewed by Fregnac (1987), it appears that role of noradrenaline in relation to visual cortical plasticity needs to be reexamined.

Serotonin has also been observed to influence the functional properties of relay cells in the LGN (Kemp at al. 1982). In the present study serotonin fibres are found to innervate the LGN from the optic tract during early gestation. These fibres become less at later gestational age. Fetal grafts from the raphe region into the fourth ventricle are found to produce variety of changes in cell migration, foliation as well as cellular architecture in the developing rat cerebellum (Yamamato et al. 1980).

It is possible that both noradrenergic and serotoninegic fibres have neurotropic effects in early stages of development. It also appears that activity-dependent autoshaping selection processes are not solely dependent on the interactions between specific afferent inputs (retinal and cortical) and the target neurons but also on the central modulatory systems such as cholinergic, noradrenergic and serotoninergic inputs. It seems that successive chemical messengers are produced at specific time periods during development which influence the establishment of the neuronal circuitry.

References

Bear M F, Kenneth M C and Ebner F F 1984 Postnatal changes in the distribution of acetylcholinesterase in kitten striate cortex; *J. Comp. Neurol.* **237** 519-532

Brecha N, Hendrickson A, Floren I and Karten H J 1982 Localisation of substance P-like immunoreactivity within the monkey retina; *Invest. Ophthalmol. Vis. Sci.* **23** 147-153

Brecha N, Johnson D, Lieberman A R and Parnavelas J 1986 Central projections of substance P containing ganglion cells in the rabbit retina; *Soc. Neurosci. Abstr.* **12** 1036

Chun J J M, Nakamura M J and Shatz C J 1987 Transient cells of the developing mammalian telencephalon are peptide immunoreactive neurons; *Nature* **325** 617-620

Coyle C T and Enna S J 1976 Neurochemical aspects of the ontogenesis of GABA ergic neurons in the rat brain; *Brain Res.* **111** 119-133

Dames W, Joo F, Feher O, Toldi J and Wolff J R 1985 γ-aminobutyric acid enables synaptogenesis in the intact superior cervical ganglion of the adult rat; *Neurosci. Lett.* **54** 159-164

De la Torre J C and Surgeon J W 1976 A methodological approach to rapid and sensitive monoamine histofluorescene using a modified glyoxylic acid technique: The SPG method; *Histochemistry* **49** 81-93

Dunner H, Von Euler V S, and Pernon B 1954 Catecholamines and substance P in the mammalian eye, *Acta Physiol. Scand.* **31** 113-118

El Badawi A and Schenk A E 1967 Histochemical methods for separate, consecutive and simultaneous demostration of AChE and norepinephrine in cryostat sections; *J. Histochem. Cytochem.* **15** 580-588

Fitzpatrick D, Penny G R and Schmechel D E 1984 Glutamic acid decarboxylase-immunoreactive neurons and terminals in the lateral geniculate nucleus of the cat; *J. Neurosci.* **4** 1809-1829

—— and Diamond I J 1980 Distributions of acetylcholinesterase in the geniculostriate system of Galago Senegalensis and Aotes trivirgatus: Evidence for the origin of the reaction product in the lateral geniculate body; *J. Comp. Neurol.* **194** 703-719

Fregnac Y 1987 Cellular mechanisms of epigenesis in cat visual cortex; in *Imprinting and Cortical Plasticity*: Comparative Aspects of Sensitive Periods. pp. 221-226 eds J P Rauschecker and P Marler (New York: Wiley and Sons)

Gabbott P L A, Somogyi J, Stewart M G and Hamori J 1986 Neurons in the dorsal lateral geniculate nucleus of the rat: characterisation using a combination of Golgi-impregnation and GABA immunocytochemistry; *Exp. Brain Res.* **61** 311-322

Hubel D H 1960 Single unit activity in lateral geniculate body and optic tract of unrestrained cats; *J. Physiol. (Lond)* **150** 91-104

Kemp J A, Roberts H C and Sillito A M 1982 Further studies on the action of 5 hydroxytryptamine in the dorsal lateral geniculate nucleus of the cat; *Brain Res.* **246** 334-337

Kostovic I and Rakic P 1984 Development of peristriate visual projections in the monkey and human fetal cerebrum revealed by transient cholinesterase staining; *J. Neurosci.* **4** 25-42

Krnjevic K and Silver A 1966 Acetylcholinesterase in the developing forebrain; *J. Anat. (Lond.)* **100** 63-69

Kuljis R O, Krause T E and Karten H J 1984 Peptide-like immunoreactivity in anuran optic nerve fibres; *J. Comp. Neurol.* **226** 222-237

Kvale I, Fosse V M and Fonnum F 1983 Development of neurotransmitter parameters in lateral geniculate body superior colliculus and visual cortex of the albino rat; *Dev. Brain Res.* **7** 137-145

McDonald J K, Speciale S G and Parnavelas J G 1981 The development of glutamic acid decarboxylase in the visual cortex and the dorsal lateral geniculate nucleus of the rat; *Brain Res.* **217** 364-367

Montero V M and Zempel J 1985 Evidence for two types of GABA containing interneurons in the A-laminae of the cat lateral geniculate nucleus: a double label HRP and GABA immunocytochemical study; *Exp. Brain Res.* **60** 603-609

Nakai Y and Takari S 1974 Influence of norepinephrine containing neurons derived from the locus coeruleus on the lateral geniculate neuronal activities of cat; *Brain Res.* **71** 47-60

Parnavelas J G and Blue M E 1982 The role of the noradrenergic system on the formation of synapses in the visual cortex of the rat; *Dev. Brain Res.* **3** 140-144

—— and Cavanagh M E 1988 Transient expression of neurotransmitters in the developing neocortex; *TINS* **11** 92-93

Pasquier D A and Villar M J 1982 Specific serotoninergic projections to the lateral geniculate body from the lateral cell groups of the dorsal raphe nucleus; *Brain Res.* **249** 142-146

Reubi J C and Jessel T M 1978 Distribution of substance P in the pigeon brain, *J. Neurochem.* **31** 359-361

Robertson R T, Hanes M A and Yu J 1988 Investigation of the origins of transient acetylcholinesterase activity in developing rat visual cortex; *Dev. Brain Res.* **41** 1-23

——, Tijerina A A and Gallivan M E 1985 Transient pattern of acetylcholinesterase activity in visual cortex of the rat; Normal development and the effects of neonatal monocular enucleation; *Dev. Brain Res.* **21** 203-214

Robson J A 1983 The morphology of corticofugal axons to the dorsal lateral geniculate nucleus in the cat; *J. Comp. Neurol.* **218** 89-103

Rogawski M A and Aghajanian G K 1982 Activation of lateral geniculate neurons by locus coeruleus or dorsal noradrengic bundle stimulation: Selective blockade by the alpha-l-adrenoreceptor antagonist parazosin; *Brain Res.* **250** 31-39

Sillito A M and Kemp J A 1983 The influence of GABA ergic inhibitory processes on the receptive field structure of X and Y cells in cat dorsal lateral geniculate nucleus (LGN); *Brain Res.* **277** 63-78

Toldi J, Farkas Z, Feher O, Dames W, Kasa P, Gywckovits K, Foo F and Wolff J R 1986 Promotion by sodium bromide of functional synapse formation from foreign nerves in the superior cervical ganglion of adult rat with intact preganglionic nerve supply; *Neurosci. Lett.* **69** 19-24

Vidyasagar T R 1984 Contribution of inhibitory mechanism to the orientation sensitivity of cat and LGN neurons; *Exp. Brain Res.* **55** 192-195

Wadhwa S and Bijlani V 1988 Cytodifferentiation and developing neuronal circuity in the human lateral geniculate nucleus; *Int. J. Devl. Neurosci.* **6** 59-75

——, Rizvi T A and Bijlani V 1988a Substance P immunoreactivity in the developing human retinogeniculate pathway; *Neurosci. Lett.* **89** 25-30

——, Takacs J, Bijlani V and Hamori J 1988b Numerical estimates of GABA immunoreactive neurons in the human lateral geniculate nucleus in prenatal period; *Human Neurobiol* **6** 261-272

Wilson J P and Hendrickson A E 1985 Electron microscopic analysis of serotoninergic axons in the monkey's lateral geniculate nucleus; *Invest. Ophthalmol. Vis. Sci. Suppl.* **26** 164

Winder A F and Patsalos P N 1974 Substance P and retinal transmission; *Biochem. Soc. Trans.* **4** 1260-1261

Yamamoto M, Chan Palay V, Steinbusch H M W and Palay S L 1980 Hyperinnervation of arrested granule cells produced by the transplantation of monoamine containing neurons into the fourth ventricle of rat; *Anat. Embryol. (Berl.)* **159** 1-15

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 17-28 (1990)

Spatial Orientation of Cells and Intercellular Interactions in Neuro-ontogenesis

N N. KOKINA
P K Anokhin Institute of Normal Physiology, USSR Academy of Medical Sciences, Moscow (USSR)

The dynamics of cell processes of the sensorimotor cortex of newborn rats in neural tissue culture revealed that individual processes had two types of growth: directed (rectipetal) and adaptive (twisting). The replacement of one type of growth with another can take place all along one neuroblast process. The adaptive and directed growths, regulated by different levels of protein synthesis have different chemical requirements.

The cell body transfer in the periods of migration and stabilization of cell position, the number of processes in pericellular zones, the relative constancy of the process number per cell, and the individual variability of neuropil density in the pericellular zone were observed.

The redistribution of cytoplasm among different parts of the network with hourly rhythm reflected neuroblasts interaction. The daily rhythms reflected glial elements activity. The many day rhythms reflected age waves of cell migration.

Cells with identical rhythms of fluctuation process number occupied a definite zone in the field of culture growth.

The question of cellular biorhythms being able to determine the spatial dispostion of cellular groups and the interaction among cells as well as between the nervous tissue transplants and the hosts tissue is discussed.

Key Words: Neuroontogenesis, Neuropil, Biorhythm, Cell, Topography

Introduction

The generally known pattern of basic stages of neuroontogenesis, including cell division, their migration and the systems orientation within the organism and relative to each other (Maximova 1979, Kokina 1980) has not so far explained the factors determining the final morphology of brain regions providing the finite function implementation.

It is known that during the structure formation, cells actively transfer and react to the environmental changes. However, the main principle underlying cellular systems development is not known so far, which could have otherwise thrown some light in solving problems facing neuroontogenesis as well as for the central nervous system regeneration and for studying the interaction of a transplant with the host's tissue.

On observing the amazing processes of forming cellular systems out of heterogenous elements in tissue cultures, we decided to carry out a thorough characterization of individual cells out of a set. This was aimed to find cellular parameters responsible for the spatial grouping of cells

Materials and Methods

One of the suitable methods of studying cellular behaviour in neuroontogenesis is the *in vitro* nervous tissue culture method (Veprintsev 1988). The sensorimotor cortex of newborn rats of Wistar line was

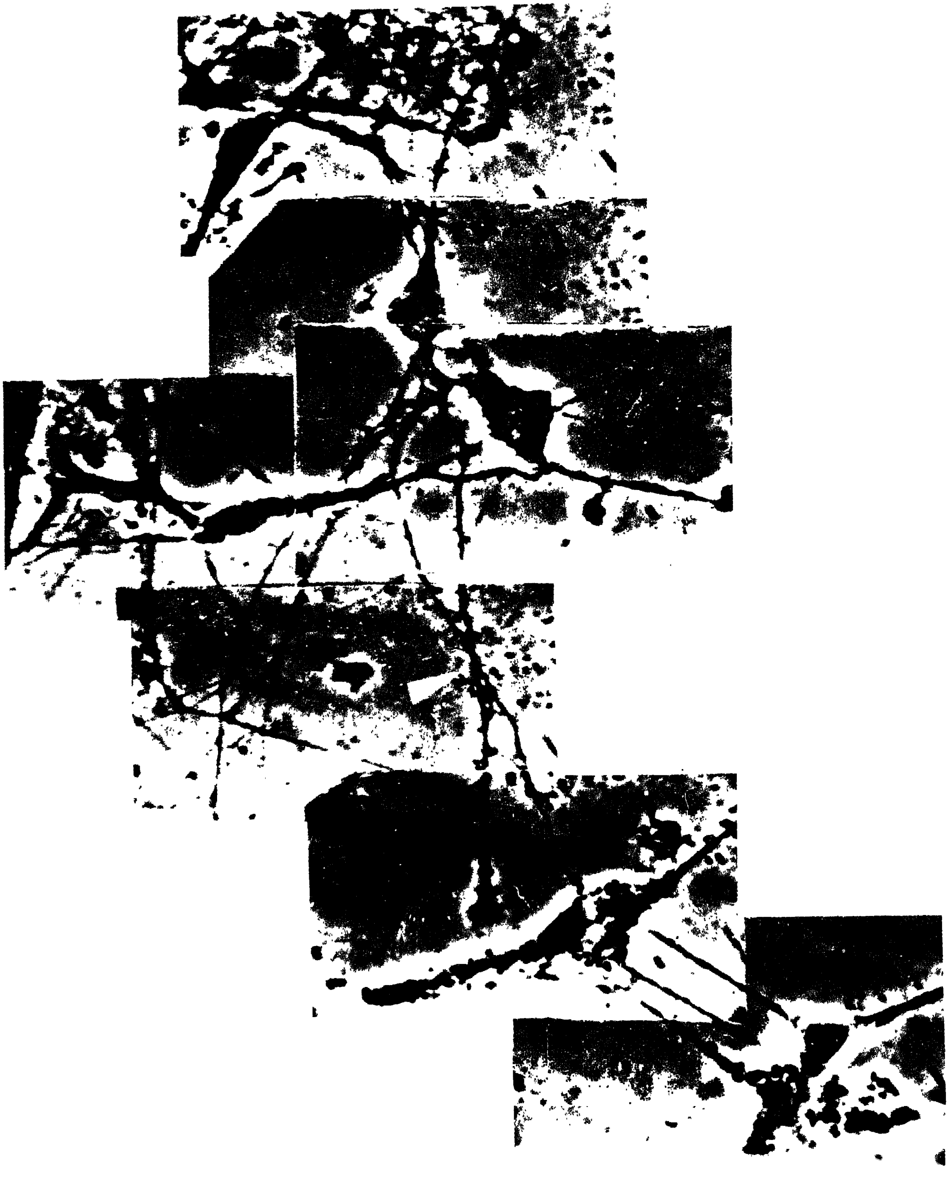


Figure 1 Phase contrast micrograph showing the neuronal-glail network in culture *in vitro* White arrows mark two types of cellular processes growth (20 × 40)

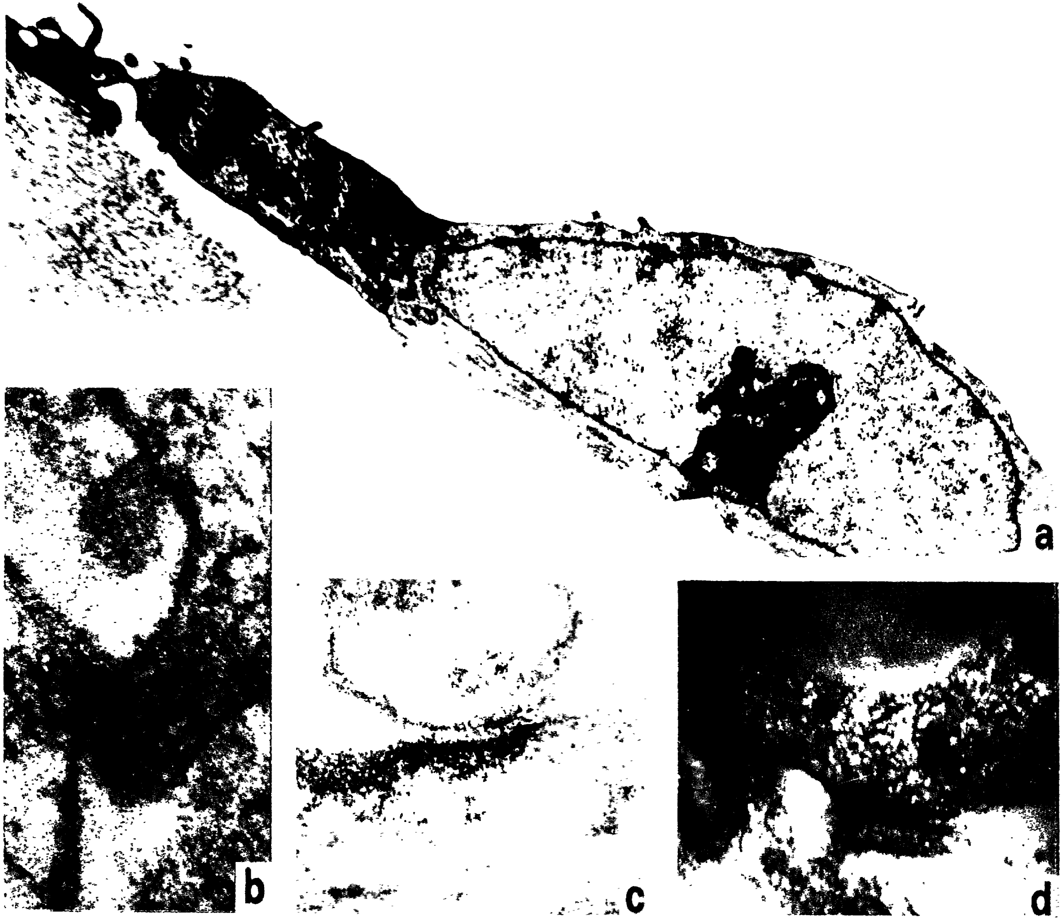


Figure 2 a-d Neuroblast (a×250) and immature contact membrane [b, c×9000; **d** ×1500]. Election microscopy data.

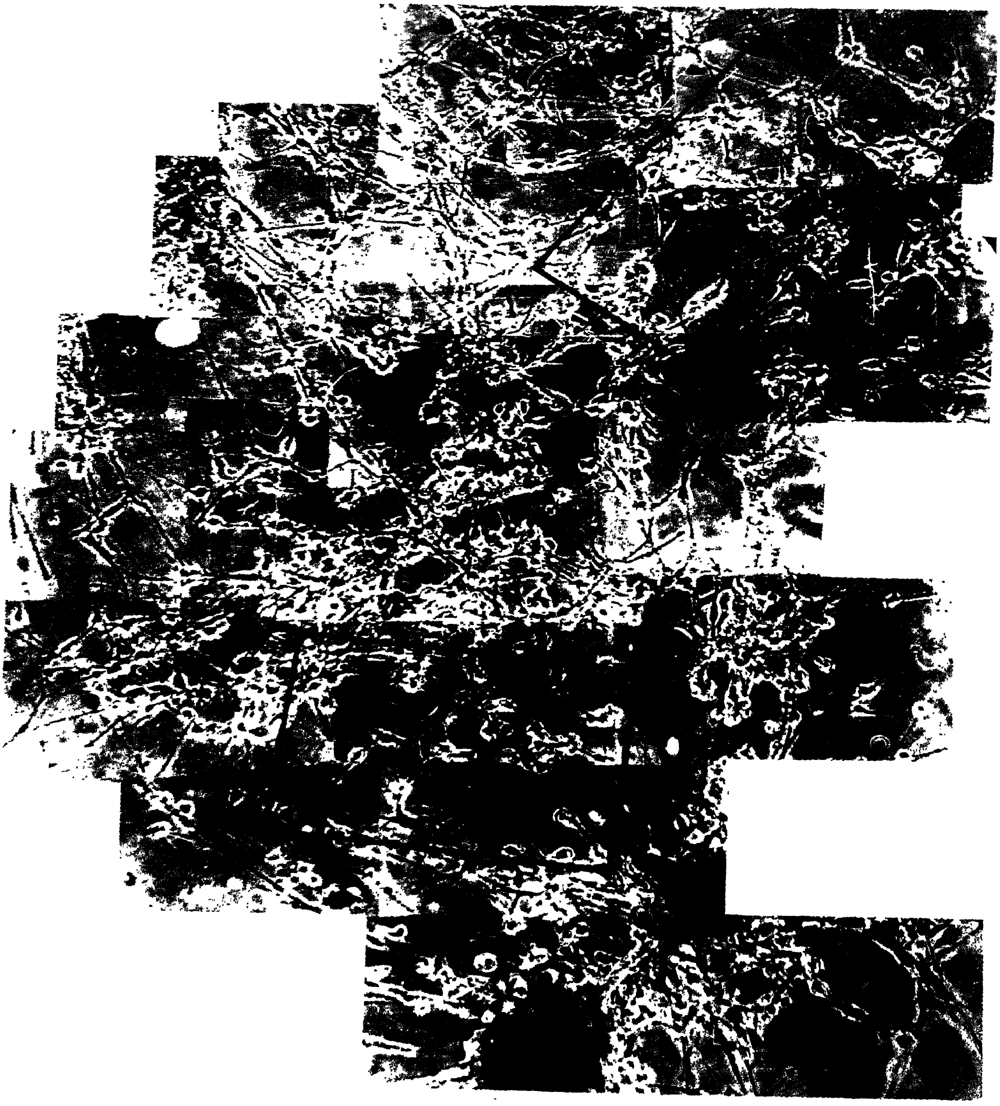


Figure 3 Phase contrast micrograph showing Photomontage of cellular network. Standard cell fields are shown in frames (10 × 20)

studied. Dissociated culture diluted by a weak (0.025%) trypsin solution was prepared to obtain single cells and small conglomerates (micro explants). It is known that the sensorimotor cortex in the intact brain of newborn rats consists of immature neuroblasts. The contacts among neuronal cells are respresented as single synapses (Maximova 1985).

In the tissue culture one can observe cell transfer and assortment by selective neighbourhood principle. In this case a neuronal-glial network preceding the consolidation of cells into more localized clusters called "units of migration" or into denser multilayer conglomerates will be the most primitive form of cellular organization. Thus, various stages of neuronal organization can be observed in tissue culture (Victorov 1985). However, it is the neuronal-glial network that, thanks to its sparse nature, will give the best possibilities for studying cellular behaviour and intercellular interactions (figure 1). It is known that such a form of nervous tissue organization is immature ontogenetically and the most ancient phylogenetically (Koshtoyants 1957).

Electron microscopic data show that in the networks of the cortical tissue culture of newborn rats the ratio of neuronal and glial elements is 1:10. The contact membranes are immature. There are single symmetric and asymmetric contacts with a few synaptic vesicles (figure 2).

The main parameter which was taken into consideration for different cells of the network was the number of processes per cell.

To take into account the dynamics of pericellular processes, the original count method developed in our laboratory by Makhmutov was applied. After taking pictures of a vast area of culture a photomontage was made (figure 3). A field of standard size including 20-30 cells with the ratio of neuronal and glial elements 1/10 was chosen for analysis. Then the middle of the greatest chord of the cell was taken for a center of a circle and was drawn with the radius calculated according to the equation

$$R = \frac{2(d_1 + d_2 + d_3)}{3}$$

where d_1, d_2, d_3 are the three chords of the cell body oriented on the basis of processes. All the processes crossing the circle and belonging to the cell itself as well as coming to the pericellular zone from other cells were counted (figure 4).

Alongside the number of processes in the pericellular zone, changes in cell body disposition during 24 hrs, the form of cell body, the presumable neuronal or glial nature of cells and their density per unit of the cover glass area in the given field of neural tissue culture were taken into account.

The observations were made round the clock beginning from the 3rd to the 14th day of cultivation.

Results

Beginning with the 3rd day of cultivation the organization of neuronal-glial network seemed to be relatively stable. However, slight transfer of cell bodies and changes in the form of processes were constantly taking place in the network. To investigate cellular mobility, the trajectories of cell body transfer in reference to a fixed point were taken into account (figure 5). The cells with a stable position continued to move relative to a center along the broken line with an amplitude not exceeding the size of four cell diameters and at the speed of not more than 15 $\mu m/hr$. The second group included cells (43%) with the same type of transfer but

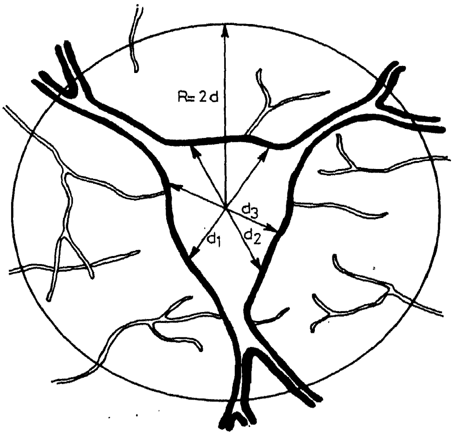


Figure 4 The scheme of the process number count in the pericellular zone R Ya. Makhmutov's method

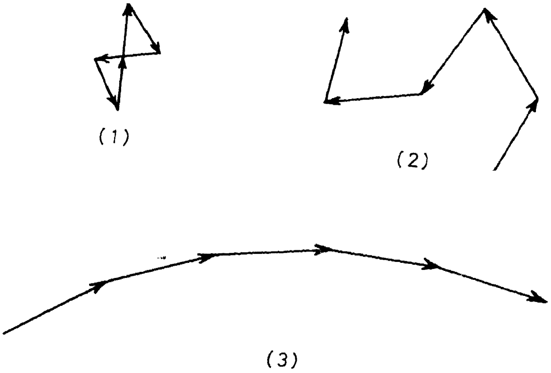


Figure 5 Different trajectories of cell migrations

with an indefinite trajectory and without a visible orientation in relation to a constant center. There was also a third type of cell (7%), moving along an arc.

The transfer speed in the second and the third cases was twice as much as that of the first type. The movement had a progressive nature. The cells of the first type which took the most stationary location continued to perform oscillatory movements. Hence, the conclusion that stabilization of cellular arrangement did not mean movement termination.

Cells with a stable arrangement were interconnected through their processes. The growth of individual processes was of two types: rectipetal and twisting. In the first case, the growth cone moved along the right line. In the second case, it performed searching movements by changing the direction, thus showing a twisting trajectory. The periods. of rectipetal and twisting growth alternated all along one process (figures 1 & 6). At the same time the cell could develop processes of one type or another (figure 7). Difference in the response to various chemicals of these two types of growth, their different functional significance and the reciprocal nature of interdependence were identified.

Ribonuclease in the concentration of 1 mg/ml added into the pericellular medium inhibited the formation of processes of both types. Desoxyribonclease in the concentration of 1 mg/ml inhibited the direction and branching of the processes. The actinomycin in the concentration of 25 μg/ml activated the formation of searching processes inhibiting the nucleus-dependent protein synthesis. The addition of ATP in the concentration of 0.1% to the pericellular medium resulted in the appearance of clusters of rectipetal processes not requiring targets. The observations compell us to assume different chemical regulation of growth of the two types of cellular processes and their potential functions.

The analysis of neuropil dynamics showed that beginning with the 3rd day of cultivation the average number of processes found in relation to one cell seemed to be constant and equalled 10. However, in the individual pericellular zones, the number of processes increased

Table 1 *The changes according to age in the dynamics of processes*

	Cultivation (days)			
1	3	6	8	14
Average number of processes per one cell	10	10	8	10
The total dynamics of processes per one cell for 4 hr	1-0.4	0.4-0.1 $P \geq 0.999$	1.3-1.0 $P \geq 0.99$	2.5-1.2 $P \geq 0.95$
% cells with,				
no change in the proceses' number	38	70	43	0
decrease in the processes' number	27	15	43	63
increases in the processes' number	35	15	14	37

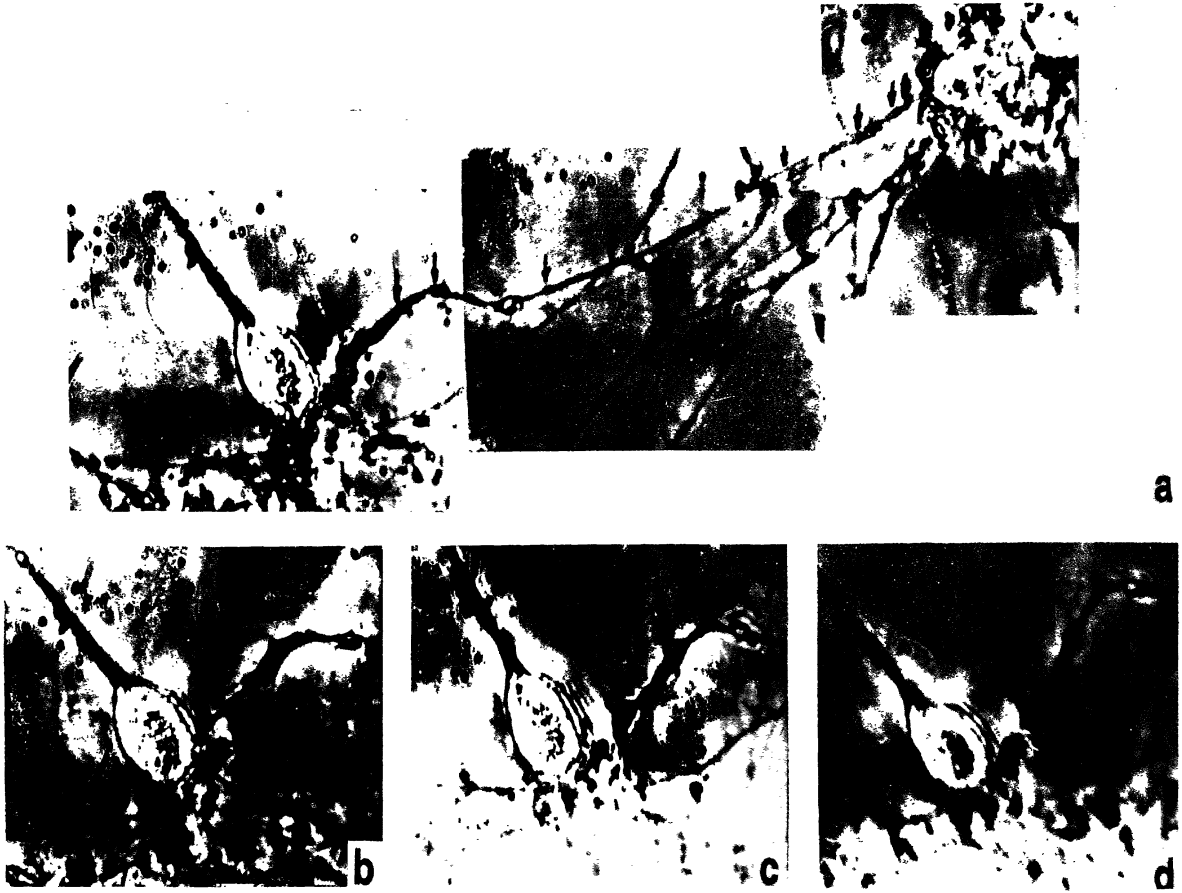


Figure 6 Interaction of two neuroblasts (1 : 2) in the tissue culture. **a**, General picture; **b**, **c**, **d**, Successive pictures show interaction between the process of Cell 9 and another cell (1) body (15 × 40); Phase contrast micrograph.

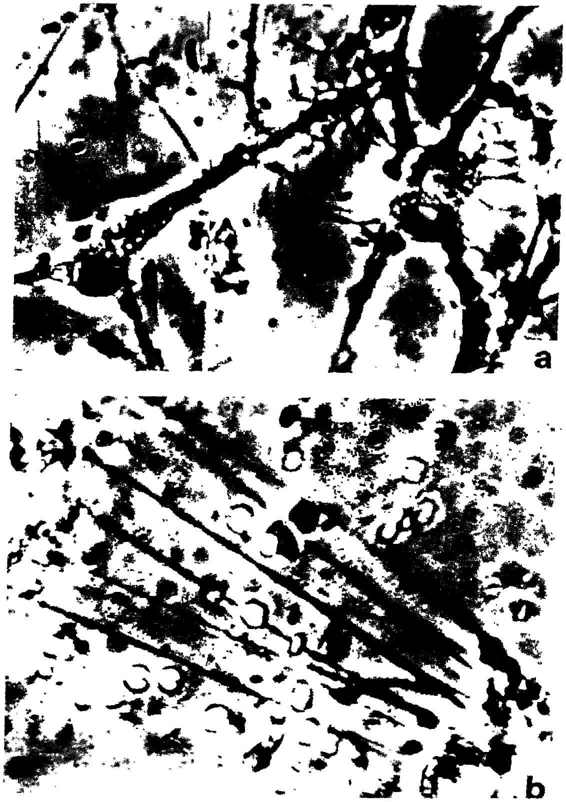


Figure 7a. The directed growth of cell processes in the nervous tissue culture stimulated by ATP; **b**, The adaptive cellular processes in the nervous tissue culture (15×40) Phase contrast micrograph

or decreased by 2-8. The degree of their activity changed with age. In a 3-day culture, one-third of cells did not manifest neuropil dynamics and in a 6-day culture there were already two-thirds of them. In an 8-day culture, the neurpil activity increased again and the cells changed the number of processes with time (table 1). The dynamics of activation in the neuropil is influenced by both appearance and disappearance of processes. The following regularity was observed: with the constant number of processes in the neuronal-glial network the cytoplasm transfer among different parts of neuropil took place. It manifested itself in the appearance of cellular processes and their disappearance from the pericellular zones of individual cells of the given network.

The dependence of neuropil dynamics on cellular density was observed—the higher the cellular density the less active the dynamic processes in the pericellular zone (table 2). One hundred and twenty individual cells were analysed for the changes in the number of process within 4 hr period. It showed that the change in the number of processes in the pericellular zone of individual cells had an oscillatory nature. The semiperiod of changes in the majority of cells was 4 hr, after which the number of processes in the neuropil decreased and then increased again. The second group included the cells whose process either increased by 6-4 ±. 4.3 or decreased by 5.3 ± 2.0 during 24 hr (figure 8). The analysis of the curves showed that there were cells having fluctuations in neuropil density with a full period of 8,10 20, 40 and 80 hr.

Table 2 *The dependence of the processes' dynamics on the cell density*

The number of cells in a standard field (n)	The change in the number of processes per cell for 5 hr
n <20	1,6 ±0,8>1
n>20	0,8 ± 0,2≥1 P≥0,99

If we correlate the disposition of cellular elements the changes in the neuropil density at different periods with reference to a stable microexplant, one would see that these elements had a definite topography of their distribution over the area with the radius of 250 μm. Cells with neuropil dynamics in the pericellular zone lasting many days were the closest to the explant. Cells with hourly dynamics were farther. And the farthest were the cells with daily pericellular neuropil dynamics. Therefore, there was a distinct intercellular interaction of individual cell groups, determining their topographical disposition. The cells concentrated within

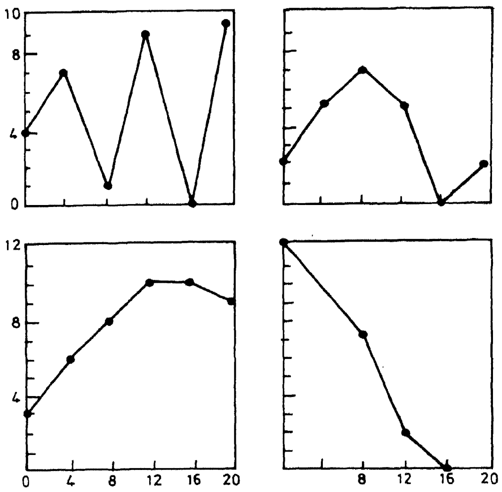


Figure 8 Different forms of changes in the process number in the pericellular zone of individual cells

X-axis—time in hours
Y-axis—the number of processes

a definite zone had common biorhythms (figure 9). As stated above, this could be judged by rhythmical fluctuations of neuropil density in the pericellular zone.

Discussion

All such events as are connected with active transfer of cells or their processes can be defined as cellular behaviour. What factors determine the cellular behaviour at a given moment? Some of them are well known. They are: mechanical tension caused by the substrate, the adhesive property of cells towards the substrate and chemical gradients of different nature. Some of the gradients are produced by neurosecretion of individual cells or cellular groups and can cause positive or negative hemotaxis of other cells and their processes. The third important factor is energetic fields existing in a living structure. First of all, they are electric fields but there can also be energetic fields of practically unknown nature, acting, for instance, by holographic principle (Shuleikina 1985, Jacobson 1978).

Besides, there is also genetic potency of a cell determining its morphology and the direction of cell transfer (Kokina 1974). Interacting by Vector Principle, intracellular and extracellular factors create a resultant determining the final position of a cell and its links with other cells (figure 10). At present comparison of external and internal factors in neuroontogenesis loses its significance and makes way for the cognition of mechanisms of their resulting interaction.

The resulting behaviour from moment to moment depends not only on the whole complex of factors but on their distribution in time. The growth of individual processes of cells of the neuronal-glial network is regulated in different periods of time from different levels of protein synthesis. The rectipetal processes are to a greater degree connected with the nucleus-dependent syntehsis, they are relatively indifferent to

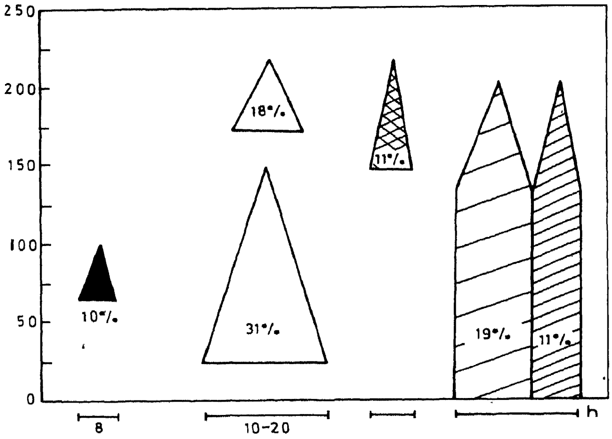


Figure 9 Grouping of cells in the growth zone of the explant in X = axis: the length of fluctuation period of neuropil density in hours; the number of cells with a given length period is expressed in percent. Y = axis: Distance from the micro-explant (μm)

external influences and to the presence of the target. They have considerable length and require high energy. The blocking of the route of cellular behaviour regulation activates the membrane-ectoplasmatic level of regulation and results in the appearance of twisting processes highly sensitive to the substrate, gradients and to the presence of adjacent cells. The change in growth type of one process means that the period of directed behaviour is followed by that of its adaptive correction.

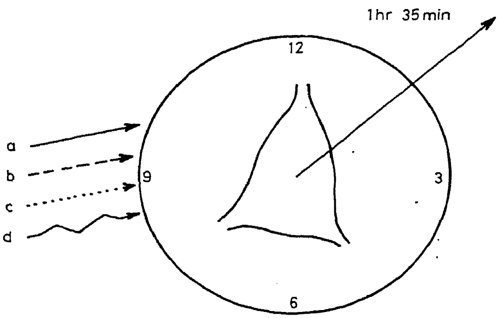


Figure 10 The vector principle of cellular behaviour determination. The vector forces influencing the cell and determining the cellular behaviour are indicated by the arrows on the left: **a,** external gradients; **b,** cell competence; **c,** cell genetic programme; **d,** cellular biorhythms. The arrow on the right indicates the resulting vector of cellular behaviour for a given moment. The process is considered relative to time.

The significance of rhythm factor for nervous tissue organization also becomes apparent following total analysis of neuropil activity in the pericellular zone. Three different intervals of neuropil pulsation are singled out: hourly rhythms (8 hr), daily rhythms (20 hr) and manyday rhythms (upto 80 hr). Hourly rhythms coincide in their characteristics with periods of change of directed adaptive growth of neuroblasts processes (figure 6). At the speed of the process growth upto 40 μm/hr during a period of several hours one can watch the development of the process into the zone of cell recipient influence, the process orientation and its leaving the zone in case the contact is not formed (figure 6). Thus, the hourly rhythms of neuropil pulsation should be related to the contact formation among neuroblasts. Cells with hourly rhythms of neuropil fluctuations are localized in a definite area of the growth zone. These cells as well as other cellular clusters can be surrounded by cells with daily rhythms. Daily variations of process activity can be characteristic of glial cells. Many day-rhythms are close to the age changes of process dynamics and they can reflect the alternation of migration and stabilization phases of cell bodies.

As the fluctuations in the number of and the type of growth are determined by the protein synthesis, the cell becomes the regulator of its own neuropil. The genetically determined biorhythm of a given cell specifies its spatial disposition in relation to other cells of the given cerebral area and the approach of other cell processes to it.

Table 3 *The distribution of cellular groups in with reference to the microexplant according to the process dynamics in the pericellular zone*

The period length of process fluctuations in the pericellular zone (hr)	Total cell No. (%)		Distance from the microexplant in arbitrary units (2.5 μm)	
			all cells within the groups	the majority of cells
80	35		0-70	0-30
40	13		60-90	
10-20	42	a) 27	10-50	
		b) 15	60-80	
8	10		23-45	

The interaction of individual biorhythms of cells is shown as one of the factors of spatial neurogenesis. In case of neural tissue transplantation the preliminary analysis of biorythms of the transplant and the host's brain in conditions of tissue culture may be used for slection of the transplanted tissue.

References

Jacobson M R 1978. *Development Neurobiology* (New York: Plenum Press)

Kokina N N 1974 Systemogenetic behaviour and maturation of nervous elements in tissue culture; *Successes of Physiological Sciences* (in Russian) **5** 93-127

——1980 Genetic determination of functional systems; In *Systemogensis* pp 26-59 ed. K V Sudakov (Moscow: Medicine)

Koshtoyants Kh S 1975 *Foundation of Comparative Physiology* (Moscow: Science)

Maximova E V 1979 *Functional Maturation of Neocortex in Prenatal Ontogenesis* (Moscow: Science)

——1985. Basic stages of neuronal cell differentiation: in *Neuroontogenesis* pp 6-76 (Moscow: Science)

Shuleikina K V 1985 *Neuroontogenesis* (Moscow: Science)

Veprintsev B N (ed.) 1988 *Methods of Neuronal Tissue Cultivation* (Moscow: Science)

Victorov I V and Shashkova N A 1986 Glio-neuronal aggregates on collagen gel; *Bull. Expl. Biol. Med.* In (Russian) 611-614

Proc. Indian natn. Sci. Acad. **B56** No. 1 .pp. 29-36 (1990)

Effect of Neonatal Undernutrition on the Brain

CHITRA SARKAR*, SUBIMAL ROY* and GOMATHY GOPINATH**
Departments of Pathology and Anatomy**, All India Institute of Medical Sciences, New Delhi 110029 (India)*

The effect of undernutrition on the maturation of the pyramidal neurons of the motor cortex and the Purkinje cells of the cerebellum was investigated on the 10th, 15th, 20th and 40th postnatal days in rat. Undernuturition was induced by increasing the litter size and reducing the suckling period. The neuronal morphology was studied by routine light and electron microscopy as well as Golgi and E-PTA stains.

The brain weight was reduced in all undernourished animals. The pyramidal neurons and the Purkinje cells in undernourished rats showed a reduction in the neuronal volume and extent of dendritic arborization on the 10th, 15th and 20th postnatal days. Though there was a gradual increase in the spine and synaptic counts in the undernourished animals, their rate of increase was very slow so that on any of the above given days, their number was significantly lower than that of the controls. In the cerebral cortex where the study was extended upto the 40th postnatal day it was found that the neuronal volume and spine count had reached values comparable to the controls but the distribution pattern of the spine count was different in the undernourished animals. Rehabilitation after the first 10 days of undernutrition showed re-establishment of normal growth of the pyramidal neurons by the 40th day. However rehabilitation after 15 and 20 days did not result in complete recovery.

These findings suggest delay in neuronal maturation in undernutrition. However, if the rehabilitation is instituted early during the postnatal period a significant recovery of the brain growth is seen. On the other hand if rehabilitation is delayed, then the chances of recovery become less. Thus the first 10 days of postnatal life appear to be the 'critical period' for neuronal maturation in this model.

Key Words: Undernutrition, Brain, Dendritic branching, Spine Count, Synapses, Rehabilitation.

Introduction

The term Protein-Calorie Malnutrition was first proposed in 1962 by the Sixth FAO/WHO Joint Committee to cover the whole spectrum of protein and calorie deficiency. The extreme degree of such deficiencies are termed as Kwashiorkar for protein deficiency and Marasmus for calorie deficiency respectively. In practice however, it is quite common to find cases with considerable overlap.

The occurrence of malnutrition is perhaps only the tip of an iceberg and the magnitude of the problem appears to be much greater. For every frank case of malnutrition there are many more cases of mild or moderate malnutrition. Though undernutrition is a global problem, it is especially so in the developing countries like India. According to the Nutrition News of 1981, 85% of pre-school children in India show some evidence of malnourishment. Recognition of even milder forms of 'undernutrition' in infants and children is extremely important since this group is unusually vulnerable to the ill effects of nutritional deprivation.

The effect of undernutrition can indeed be a serious one since a large body of accumulated evidence indicates that it can have profound adverse effects on many organs of the body (Deo et al. 1965). Perhaps the most important effect is on the development and maturation of the brain. While the relative resistance of the adult brain to nutritional deprivation is a well established phenomenon (Winick & Noble 1966, Dobbing 1968) undernutrition in early life during the "critical period"or "vulnerable period" of brain development can produce widespread structural and functional changes (Winick & Noble 1966, Dobbing 1968). In human studies reduced head circumference, low intelligent quotient (IQ) for age, impairment of abstract thinking and poor neurointegrative functions have been observed in various studies (Stoch & Smythe 1967, Champakan et al. 1969, Agarwal 1984).

To elucidate the structural basis for the above behavioural and functional alterations, experiments have been designed in animal models by many investigators. Rat has been chosen as the ideal model since it has the same sequence and pattern of brain development as that in the human though the time period differs. In rodents, the brain development extends from the prenatal period to first 3 weeks of the postnatal life while in human beings it extends upto the first 8 years from the prenatal life. Brain development can be divided into 2 phases-pregrowth spurt phase and growth spurt phase Dobbing (1970) proposed that brain grows especially rapidly over a sharply defined period of time referred to as growth spurt period. Period of brain growth spurt is the 'critical period' or 'vulnerable period' (Dobbing 1970, Dobbing and Sands 1971) when brain is maximum susceptible to nutritional insult. This phase lasts from 13th week of gestation to 2-3 years of age in humans while the corresponding phase is mainly postnatal in rats (Dobbing 1970). During the pre-growth spurt phase, neuronal proliferation occurs and once neuronal proliferation has ceased and the adult number of neurons has been obtained, the growth spurt period starts.,The early part of this growth spurt period is concerned mainly with glial cell multiplication and the later part with myelination, development and growth of axons and dendrites and elaboration of synaptic connections.

Developing cerebellum in the rat has been extensively studied to determine the effect of undernutrition on all three phases of neuronal growth, proliferation, differentiation, and maturation, which occur in the first 3 weeks of postnatal life unlike in cerebrum where the entire process of neuronogenesis is prenatal. Further, from 28 weeks of gestation to one year of postnatal life, the development of cerebellar cortex and the maturation of the Purkinje cells in human are comparable to cerebellar growth and differentiation in the first 3 weeks of postnatal life in the rat (Dobbing 1970).

According to Dobbing (1970), this 'critical period' is a once and for all opportunity for the brain to accomplish certain important processes of development. If this opportunity is lost, it will be permanentaly deranged and cannot fully recover. Thus "catch-up" growth can occur if nutritional rehabilitation is instituted early (Gopinath 1981). Especially if undernutrition occurs after cell division has ceased, then there is no decrease in cell number but only in cell size and this can be restored to normal if adequate nutritional supplements are given.

Most of the animal studies on undernutrition have focussed on brain weight, cell number, chemical composition and myelination (Sastry & Murthy 1984). Amount of DNA is reduced and this reflects decreased number of cells. Whole brain or regional concentration of myelin is found to be reduced. A wide range of alterations in the brain metabolism have been reported eg decreased conversion of glucose to amino acids, delayed glutamine conversion to glutamate, decreased activity of various enzymes like acetyl choline esterase etc. Increased levels of acetylcholine, serotonin and changes in other catecholamines have been observed. Some studies are also available on the alterations in the dendritic tree and synapses in undernutrition which will be of greater relevance to the functional alterations in the brain (Salas et al 1974, Salas 1980, Dyson & Jones 1976, Gambetti et al, 1974, McConnell & Berry 1978a). Results of rehabilitation studies show conflicting data about recovery (Gopinath et al, 1981, McConnell & Berry 1978b, Angula-Colmenares et al, 1979).

As undernutrition is an important problem in our country, we undertook this study to find out the effects of postnatal undernutrition on the cerebrum and cerebellum with particular reference to the development of the dendritic tree, dendritic spines and synapses which form the neuronal circuitry. Further, reports on the results of rehabilitation are scanty and conflicting.

Materials and Methods

Inbred rats of As_2 strain were used in the present study. Total calorie undernutrition was induced during the postnatal period by increasing the litter size to 16 by pooling the pups to a foster mother on an ad lib diet and by restricting the feeding time to 14 hours a day by separating the pups from the mother. Only 6 pups were allowed to a mother in the control group and they remained continuously with the mother. After weaning only 30% of the diet of the control rats was allowed to the undernourished rats. Animals were sacrificed on the 10th, 15th, 20th and 40th postnatal days by decapitation and the left frontal cortex and cerebellum were removed.

In another set of experiments, the undernourished rats were rehabilitated after 5, 10, 15 and 20 days of undernutrition. Rehabilitation was done by reducing the litter size to 6 and by keeping them continuously with the mother. After weaning, the animals had free access to the diet as in control groups. All the rehabilitated animals were sacrificed on the 40th day after birth. Only cortex was studied in the rehabilitated animals.

The brain samples were studied using routine light microscopy, Golgi staining for the dendritic tree and ethanol-phosphotungstic acid (E-PTA) staining for the synapses. After Golgi staining the Purkinje cells and pyramidal neurons were drawn with camera lucida at X 400 magnification and the characteristics of the cell body as well as order and extent of dendritic arborisation noted.

Results

Body weight

The body weight of the undernourished animals was significantly less than that of the controls on all the days (table I).

The difference in body weight was more on the 10th day compared to that on 15th postnatal day. Thereafter, the control animals gained weight rapidly while the undernourished pups grew at a slower pace till the 40th postnatal day. The rehabilitated animals gained weight irrespective of the day on which the rehabilitation was instituted but it had not caught up with the control values till the 40th postnatal day.

Brain weight

Total brain weight was also significantly less on the 10th postnatal day following undernutrition (table 1). Thereafter, the difference between the brain weights of the undernourished and control rats was considerably reduced and by the 40th postnatal day, the difference was much less. The brain weight showed catch-up growth irrespective of the day on which rehabilitation was started and had improved to a considerable extent between the 20th and 40th postnatal day.

The cerebellar weight was also reduced in the undernourished rats. Maximum reduction was noted on 10th day (table 1).

Cerebellum

In the sagittal sections, in the control cerebellum all the fissures and folia were well developed and clearly identifiable by the 10th postnatal day. On the 15th and 20th postnatal days, the folia were larger and the fissures had deepened. Following undernutrition, the total surface area of the cerebellum seen as the area occupied on the section was reduced on the 10th, 15th and 20th postnatal days. The folia were thinner and shorter and the fissures wider and shallower.

On the 10th day in the control animals all 4 layers of the cerebellar cortex were well differentiated. The external granular layer (EGL) was present and thick but internal granular layer (IGL) was thin. The molecular layer contained vertically oriented spindle cells with dark nuclei-the migrating cells. The Purkinje cells were arranged in a single regular row and they were closely packed having round vesicular nuclei with prominent nucleoli and thin concentric rim of cytoplasm, more at the apex. By the 15th postnatal day the EGL decreased in thickness while thickness of IGL and molecular layer increased, Purkinje cells had developed more basal cytoplasm.. By 20th day, EGL had completely disappeared in most parts while in others it persisted as in-

Table 1 *Body, brain and cerebellum weights of control undernourished and rehabilitated animals*
(Data, expressed as g, are mean±SD)

Postnatal (days)	Groups	Body weight (g)	Brain weight (g)	Cerebellum weight (g)
10	C	17.64±1.68	0.94±0.96	0.21 ±0.008
	U	6.86±0.78	0.52±0.05	0.13±0.004
15	C	20.83±0.87	1.19±0.08	0.26±0.006
	U	12.00±2.25	0.89±0.11	0.20±0.1010
20	C	33.23±2.68	1.31±0.07	0.30±0.004
	U	15.82±2.05	1.08±0.17	0.22±0.007
40	C	92.00±6.45	1;58±0.058	—
	U	44.5±7.68	1.40±0.03	—
5	R	87.83±7.19	1.53±0.08	—
10	R	69.00±5.46	1.41±0.11	—
15	R	77.85±10.95	1.37±0.12	—
20	R	77.57±7.09	1.5±0.07	—

C, Control; *U,* Undernourished; *R,* Rehabilitated

terrupted single layer of cells. The thickness of molecular layer and IGL had increased. There were very few migrating cells in the molecular layer. Purkinje cells were widely spaced with evenly distributed concentric rim of cytoplasm.

In the undernourished rats, the EGL was thicker on both 10th and 15th postnatal days, while molecular layer and IGL were thinner than those in controls. On the 10th day, Purkinje cells were irregularly placed, not arranged in single row and had only apical cytoplasm. By 15th day, Purkinje cells were in a single row similar to that of the their age-matched controls. On the 20th day, the EGL still persisted as 1-2 cell layers or several cell layers thick. The molecular layer was thinner with a number of dark migrating cells. The Purkinje cells on the 10th day were immature and resembled that of 7th day control rats but by 15th and 20th days, attained the morphology similar to that of controls. This indicates that maturation is occurring but it is delayed.

The Purkinje cell body was smooth and devoid of any processes, as seen in Golgi stain, on the 10th postnatal day control animals. Dendritic branching was seen upto 3rd or 4th order. But on 10th day in undernourished animals the Purkinje cell surface was spiny and perisomatic processes were observed on the cell body. These peri-somatic processes represent axo-somatic contacts of the climbing fibres during the first postnatal week. The dendritic network was much less elaborate. In 15th and 20th day controls, dendritic arborisation had increased and become more complex. In the undernourished group of the same age the cell body had become smooth, dendritic arborization had increased but was always less developed than that in the controls.

The dendritic spines in the Purkinje cells are postsynaptic elements for the afferent inputs mostly by the parallel fibres of the granule cells. In the control rats, the spines studied over a measured area (2-3 mm) of terminal dendrites showed an increase in their number till the 15th day followed by a decline by the 20th day.

In the undernourished the spine count was very low on the 10th day but increased steadily between 10 and 15 days, after which there was a rapid decline (table 2). Unlike the spine count, the synaptic density/100 micron^2 was found to be steadily increasing in both control and undernourished rats. However, the count in the undernourished group was always significantly less than in the controls (table 3).

Table 2 *Comparison of spine count per mm of terminal dendrite in control and undernourished rats*
(Data are mean ± SE)

Postnatal days	Groups	Spine count
10	C	969.5 ± 23.17 (21)
	U	487.37 ± 26.29* (18)
15	C	1194.37 ± 24.69 (40)
	U	996.97 ± 23.53* (39)
20	C	1184.04 ± 42.83 (18)
	U	910.93 ± 34.22* (16)

Figures in parentheses indicate the number of neurons studies.
C, Control; *U,* Undernourished
*In all the three groups the difference in spine count was statistically significant ($P < 0.001$)

Table 3 *Comparison of synaptic count per 100 micron^2 in control and undernourished rats in cerebellum*

Postnatal days	Groups	Synaptic count
10	C	7.68 ± 0.627
	U	Nil in some areas and 1 or 2 in others*
15	C	12.79 ± 1.42
	U	8.36 ± 0.916*
20	C	20.04 ± 1.27
	U	9.74 ± 0.617*

C, control; *U,* undernourished.
*In all the three groups, the difference in synaptic count between control and undernourished animals is statistically significant ($P < 0.001$).

Cerebral Cortex

No change was observed in the cortical thickness or layering in any part of the cerebral cortex after postnatal undernutrition. The pyramidal neurons in the 3rd layer of the supra-granular cortex are considered to be more important for the higher functions and develop later than the other layers. Therefore, they are likely to be affected more by undernutrition and were thus chosen for our study.

There was marked decrease in the dendritic growth of the pyramidal neurons resulting in decrease of the entire neuronal volume till the 20th postnatal day of undernutrition. On the 40th postnatal day, no apparent difference was seen inspite of the undernutrition. Neuronal size and volume of the rehabilitated animals were comparable with that of the controls of the 40th postnatal day.

Since the apical dendrite is the first to develop and acquire spines, a segment, a few microns distal to the cell body was chosen to count the spines. The spines on the apical dendrites of the medium sized pyramidal neurons developed by the 10th postnatal day and there was a rapid increase in their number between 10th and 20th postnatal days (table 4). However, by the 40th day there was a fall. The proximal segment of the apical dendrite had spines till the 20th postnatal day but on the 40th postnatal day, this segment was spine free or there were only short stubby spines, while the distal regions had higher population of pedunculated spines.

In the undernourished rats, though there was marked decrease in the spine count on the 10th, 15th and 20th postnatal days, they continued to increase till the 40th day. By the 40th day, the count was almost same as in the control animals, but the distribution of the spines and their morphology were like those in the 20th postnatal day of control. Spines were still present in the proximal segment of the apical dendrites in the undernourished animals of the 40th day.

There was no difference in the spine count of animals rehabilitated after 5 and 10 days of undernutrition and the controls of 40th postnatal day i.e. they had reached normal values (Table 4). But the animals rehabilitated

Table 4 *Spine count in the control, undernourished and rehabilitated animals in cerebral cortex.* (Data are mean±SD)

Batch		Days 10	 15	 20	 40
Control		2.909 ±1.465	12.9655 ±3.986	28.365 ±3.94	21.796 ±6.6655
Undernourished		1.074 ±1.035*	2.57 ±1.267*	11.634 ±5.69*	22.1 +3.92
Rehabilitated	22.54 +4.85	20.78 +3.67	25.38 +4.35	29.39 +7.54**	

P as compared to control, *<0.001; P as compared to 40 day control, **<0.01

Table 5 *Synaptic density in control, undernourished and rehabilitated animals in cerebral cortex* (Data are mean±SD)

Batch	Days 10	 15	 20	 40
Control	5.74±1.08	11.94±3.49	13.01±3.96	22.37±4.11
Experimental	3.26±1.22	3.77±1.43	5.38±2.02	13.95±2.47
Rehabilitated	25.48±3.76	14.97±5.16	11.52±1.42	

after 15 and 20 days of undernutrition had low spine count and were nearer to that of the 20th day control animals. The number of spines in the proximal segment of the apical dendrite was more in the animals rehabilitated after 20th day than those rehabilitated after 15 days of undernutrition. Since we have not conducted any studies beyond this period. it is not possible to assess the final status of the spines in the rehabilitated rats.

In both the control and undernourished animals, the number of synapses showed a gradual increase but the increase was much less in the experimental groups. The difference in synaptic count between the control and undernourished animals was statistically significant (table 5).

On rehabilitation, the number of synapses increased and reached almost the normal level in animals rehabilitated after 10 days of undernutrition but remained significantly low in the animals rehabilitated after 20 days of undernutrition. The synaptic density fell in the intermediate range in animals rehabilitated after 15 days of undernutrition.

Discussion

The marked effect of undernutrition on the body weight compared to the total brain weight confirms the relative 'brain sparing' during development compared to other tissues as has been reported earlier (Winick & Noble 1966).

The thicker EGL and its persistence for a longer period in the undernourished animals indicates delay in migration of the EGL cells to the IGL (Sima & Persson 1975, Gopinath et al. 1976, Clos et al. 1977, Lewis et al. 1975). This is further confirmed by the greater number of migrating cells in the molecular layer of the undernourished rats even on 20th postnatal day. Further, it is likely that as a result of delayed differentiation and migration of EGL cells, there may be retardation in the formation of the basket, stellate and granule cells in the molecular layer and IGL. This is confirmed by the thinner molecular layer and IGL. Decrease in the granule cell number has been reported by Neville and Chase (1971), Barnes and Altman (1973) and Clos et al. (1977).

It is evident from this study that undernutrition markedly delays the maturation of Purkinje cells. The persistent peri-somatic processes in the undernourished rats of the 10th day indicate that their resorption is delayed in undernutrition (Chowdhury et al. 1982). Purkinje cells are formed prenatally but their dendritic elaboration and maturation are postnatal which are adversely affected by postnatal undernutrition. Dendritic elaboration of the Purkinje cells depends mainly on the afferent inputs, mostly from the parallel fibres which are the axons of the granule cells (Vaughn et al. 1974). Significant decrease in the granule cell population has been shown in undernutrition (Barnes & Altman 1973, Clos et al. 1977). Therefore, the loss of granule cells and consequently the parallel fibres appear to be responsible for the decrease in elaboration of the dendritic network and the formation of unusually long primary dendrite seen in some neurons (Chowdhury et al. 1982). The changes in the Purkinje cells and their dendritic growth may partly be due to the direct effect of undernutrition by decreasing the availability of nutrients and metabolites necessary for normal cell growth and development.

The initial increase in the spine count in control animals can be attributed to addition of new spines and increase in dendritic branching. According to Bradley and Berry (1976), the formation of these spines is genetically determined since spines can be formed without pre-synaptic influence. However, their maintenance requires them to establish appropriate synaptic connections. Thus the decrease of the spine count in the control animals after the 15th postnatal day may be due to resorption of the spines which fail to make contact with the parallel fibres, a process which occurs by natural selection (Larra Mendi & Victor 1966). Decline in the count may also be due to resorption of the previously developed dendritic branching, especially the terminal segments as a result of failure to develop contacts. Lengthening of dendritic segments during growth may add to the decline in spine count.

The consistently reduced synaptic count in the cerebellum of undernourished animals is in conformity with the findings of Dyson and Jones (1976). The decrease could be due to direct nutritional insult or through delayed and reduced afferent input.

Similar delay in growth and maturation of neurons and their dendrites, spines and synapses was seen in the cerebral cortex (Gopinath et al. 1981).

The results of the rehabilitation studies are very interesting. There was recovery of neuronal volume in the rehabilitated animals and this recovery directly correlated with the time at which the rehabilitation was instituted. Early rehabilitation definitely facilitates the recovery of the dendritic spines while the animals rehabilitated later are slower to recover. Only minimal recovery of the dendritic tree has been reported by McConnell and Berry (1978b) in the Purkinje cells in rats rehabilitated after 10 days of undernutrition. However, from the present study it is not clear whether the synaptic number will reach the values seen in normal adult animals.

Thus all the findings observed in the present study indicate that undernutrition adversely affects the maturation processes of the neurons in the developing brain. However, the increase in brain weight, level of dendritic branching, dendritic spines and synapses follow a pattern similar to that of the control though at a significantly slower rate, indicating a delay in the morphological maturation during undernutrition. The delay in the development of these parameters which form the basis of the neuronal connection will effect the timely development of functional and behavioural modalities, thus impairing the neurointegrative functioning. However, if rehabilitation is instituted early before the 'vulnerable period' of Dobbing, there will be rapid and almost complete reversibility of these parameters, while only slow recovery is recorded by later rehabilitation. The first 10 days of postnatal life appear to be the 'critical period' or 'vulnerable period'. 'Catch-up growth' in cerebellum cannot be commented upon since it was not studied.

Acknowledgement

The authors acknowledge the support of the WHO for funding the project. They thank the Indian Council of Medical Research and the Indian Journal of Medical Research for the permission to reproduce tables II and III from one of our earlier publications in IJMR (Vol. 75, April 1982) and tables II and III from another publication in IJMR (Vol. 74, December 1981). We also thank Miss D. Tuteja for the secretarial assistance.

References

Agarwal K N 1984 Malnutrition and mental development; in *Nutrition and Brain.* pp 58-77 eds P N Tandon and G Gopinath (New Delhi: Indian National Science Academy)

Angulo-Colmenares A G, Vauhan D W, Hinds J W 1979 Rehabilitation following early malnutrition in the rat: Body weight, brain size, and cerebral cortex development; *Brain Res.* **169** 121-138

Barnes D, Altman J 1973 Effects of different schedules of early undernutrition on the pre-weaning growth of the rat cerebellum; *Exp. Neurol.* **38** 406-419

Bradley P and Berry M 1976 The effects of reduced climbing and parallel fibre input on Purkinje cell dendritic growth; *Brain Res.* **109** 133-151

Champakam S, Srikantia S G and Gopalan C 1968 Kwashiorkar and mental development; *Am. J. Clin. Nutr. 21 844-852*

Chowdhury C, Gopinath G and Roy S 1982 Effect of undernutrition on the maturation of Purkinje cells in the rat; Indian J. Med. Res. **75** 559-566

Clos J, Favre C, Selme Matrat M and Legrand J 1977 Effects of undernutrition on cell formation in the rat brain and specially on cellular composition of the cerebellium; *Brain Res.* **123** 13-26

Deo M G, Sood S K, Ramilingaswami V 1965 Experimental protein deficiency. Pathological features in Rhesus monkeys. *Arch. Pathol.* **80** 14-23

Dobbing J 1968 Vulnerable periods in developing brain, in *Applied Neurochemistry* pp 287-295. eds. AN Davison and J Dobbing (Oxford and Edinburgh: Blackwell Scientific Publications)

Dobbing J 1970 Undernutrition and the developing brain. The relevance of animal models to the human problem. *Am. J. Dis. Child.* **120** 411-415

Dobbing J, Sands J 1971 Vulnerability of the developing brain IX The effect of nutritional growth retardation on the timing of the brain growth spurt. *Biol. Neonate* **19** 363-370

Dyson S E, Jones D G 1976 Some effects of undernutrition on synaptic development a quantitative ultrastructural study. *Brain Res.* **110** 365-378

Gambett P, Autilio-Gambetti L, Rizzuto N, Shafer B, Pfaff L 1974 Synapses and malnuttition: Quantitative ultrastructural study of the rat cerebral crotex. *Exp. Neurol.* **43** 464-473

Gopinath G, Bijlani V, Deo M G 1976 Undernutrition and the developing cereballer cortex in the rat. *J. Neuropath. Exp. Neurol.* **35** 125-135

——, Roy S, Karmarkar M G 1981 Effects of undernutrition and rehabilitation on the cerebral cortex in rat; *Indian J. Med. Res.* **74** 866-871

Larra Mendi L M H and Victor J 1966 Soma-dendritic gradient of spine resorption in the Purkinje cell of the cerebellum of the mouse during postnatal development. An electron microscopic study; *Anat. Rec.* **154** 373 (Abstr.)

Lewis PD, Balazs R, Patel A J and Johnson A L 1975 The effect of undernutrition in early life on cell generation in the rat brain; *Brain Res.* **83** 235-247

McConnell P, and Berry M 1978a The effects of undernutrition on Purkinje cell dendritic growth in the rat; *J. Comp. Neurol.* **177** 159-171

McConnell P, Berry M 1978b The effect of refeeding after neonatal starvation on Purkinje cell dendritic growth in the rat; *J. Comp. Neurol.* **178** 759-772

Neville H E and Chase H P 1971 Undernutrition and cerebellar development; *Exp. Neurol.* **33** 485-497

Salas M 1980 Effects of early undernutrition on dendritic spines of cortical pyramidal cells in the rat; *Dev. Neurosci*3 109-117

Salas M, Diaz S and Nieto A 1974 Effects of neonatal food deprivation on cortical spines and dendritic development of the rat; *Brain Res.* **73** 139-144

Sastry P S and Murthy P S V R 1984 Nutritional effects on the biochemistry of developing brain; in *Nutrition and Brain.* pp 38-57 eds P N Tandon and G Gopinath (New Delhi: Indian National Science Academy)

Sima A and Persson L 1975 The effect of pre-and postnatal undernutrition on the development of the rat cerebellar cortex. I. Morphological observations; *Neurobiol.* **5** 23-30

Stoch M B and Smythe P M 1963 Does undernutrition during infancy inhibit brain growth and subsequent intellectual development? *Arch. Dis. Childh.* **38** 546-552

Vaughn J E, Henrikson C K and Grieshaber J A 1974 A quantitative study of synapses on motor neuron dendritic growth cones in developing mouse spinal cord; *J. Cell Biol.* **60** 664-672

Winick M and Noble A 1966 Cellular response in rats during malnutrition at vaious ages; *J. Nutri.* **89** 300-306

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 37-42 (1990)

Effect of Neonatal Undernutrition on Neuromuscular System

SUBIMAL ROY* GOMATHY GOPINATH**, MADHU G KARMARKAR***, AMIT K DINDA*, and CHITRA SARKAR*
Departments of Pathology, Anatomy** and Endocrinology****
All India Institute of Medical Sciences, New Delhi 110029 (India)

The effects of undernutrition on the skeletal muscle and peripheral nerve were studied in neonatal rats on the 10th, 15th, 20th and 40th postnatal days. Undernutrition was induced by increasing the litter size and reducing the suckling time. The tissues were also studied from 40 day-old rats rehabilitated after 10, 15 and 20 days of undernutrition. Significant reduction in muscle diameter was recorded in the undernourished rats. Diameter reduction was more marked after the 15th postnatal day. Though the rats rehabilitated after 10 days showed better recovery of muscle volume, complete restoration of the muscle volume was not achieved in any of the rehabilitated groups. Normal growth pattern of the nerve fibres and myelin of the sciatic nerve was also affected as a result of undernutrition. Both axonal and myelin growth were delayed resulting in reduction in fibre diameter and myelin thickness. Significant growth retardation persisted in the nerve fibres after rehabilitation. The regeneration of the sciatic nerve following crush injury in undernourished new born rats was also studied by light and electron microscopy and by immunohistochemistry after 3,7,15,25 and 40 days of crushing. The onset and progress of nerve regeneration in the form of axonal sprouting and myelination were slower in the experimental group than in normal controls. The onset of Schwann cell proliferation was early and more intense in the control groups than in the experimental ones but it persisted for a longer time in the latter.

Key Words: Neonatal undernutrition, Skeletal muscle, Peripheral nerve

Introduction

It has been well established that in both man and experimental animals, the brain is vulnerable to damage due to malnutrition particularly at the period of its rapid growth (Dyson & Jones 1976, Dobbing 1970, Dobbing & Sands 1971). Many authors have studied different aspects of brain development in animals subjected to prenatal or neonatal undernutrition. Reduction of weight of the brain, reduction in size and number of neurons, reduced formation of myelin as well as reduction in the dendritic development and number of synapses have been reported (Salas et al. 1974, Dyson & Jones 1976, Gambetti et al. 1974, Gopinath et al. 1981, Chowdhury et al. 1982). However, studies on the effect of neonatal undernutrition on the growth of skeletal muscles and peripheral nerves have been limited (Haltia et al. 1978, Heddley-White 1973, Hedley-White & Mauser 1971, Sima 1974, Mendes & Waterlow 1958). We investigated the effect of undernutrition on the skeletal muscle and peripheral nerve of newborn rats and also studied these structures after rehabilitating them with normal diet (Gopinath et al. 1983).

Peripheral nerve injury is a common and an important clinical problem and though the effect of undernutrition on the growth of peripheral nerve has been studied by many authors, there is very little work on the effect of undernutrition on regeneration of peripheral nerve following traumatic insult. Repair is normally accompanied by inflammatory reaction in the early stage and proliferation of Schwann cells, myelination, growth of axons, etc. at the later stage. Considering that these processes may be affected by undernutrition, we also investigated the effect of neonatal undernutrition on regeneration of traumatised peripheral nerve.

Materials and Methods

Inbred AS_2 rats were used for this study. The material and methods on induction of neonatal undernutrition were same as given in details in the accompanying paper (Sarkar et al. 1990).

For the study of the effect of undernutrition on skeletal muscle and peripheral nerve, tissues from the quadriceps femoris muscle and the sciatic nerve respectively were fixed in 10% buffered formalin for light microscopy (LM) and in 2.5% glutaraldehyde fixative for electron microscopy (EM). Cross sections of muscle tissue stained with haematoxylin and eosin (H&E) were examined and the mean diameter of 100 fibres was measured for each animal. For the various measurements of peripheral nerves, ultrathin sections were examined under EM and were photographed under low power. Fibre diameter and myelin thickness of 1000 myelinated fibres from randomly distributed fields were measured in each group.

For the study on the regeneration of peripheral nerve following crush injury, the sciatic nerve was subjected to crush injury on the 20th day after birth in both control and undernourished rats and the litters were weaned on the 21st day. The animals were sacrificed on 3, 7, 15, 25 and 40 days after crushing. After weaning, the undernourished rats received only 30% of diet consumed by the control animals.

The crushed segments were fixed in 10% buffered formalin and in 2.5% buffered glutaraldehyde solution for LM and EM respectively. Paraffin sections were examined by LM after H&E stain and luxol-fast blue and Bielschowsky's silver stain for myelin and axon respectively. Paraffin sections were also used for immunohistochemical method using Avidin-Biotin conjugate (ABC) immunoperoxidase staining. Antibodies against S-100 protein, tubulin and myelin basic protein were used as markers for Schwann cells, axon and myelin respectively.

Results

Undernutrition and Skeletal Muscle and Nerve

The diameter of muscle fibres in the control rats did not show any significant increase between the 10th and 15th postnatal day, but there was progressive and rapid increase in fibre size thereafter to about two fold by the 40th postnatal day (table 1). In the undernourished animals the diameter of muscle fibres was significantly reduced and the reduction was more pronounced with the advancement of age (table 1). There was a remarkable improvement in fibre diameter in the rehabilitated rats and the measurement in rehabilitated animals after only 10 days of undernutrition was close to that in control animals (table 1). The difference in measurement was more striking in the rats rehabilitated at later periods. Apart from reduction in the fibre diameter, no appreciable structural alteration was seen in the muscle in the undernourished animals.

Table 1 *Diameter of muscle fibres in control, undernourished and rehabilitated rats*

Batch	Days			
	10	15	20	40
Control	11.80 ± 0.98	12.85 ± 1.68	18.95 ± 0.83	24.40 ± 1.80
Undernourished	9.93 ± 0.97	9.88 ± 0.30	12.71 ± 0.34	13.6 ± 0.98
Rehabilitated	23.57 ± 0.33	22.15 ± 0.18	21.06 ± 0.32	

In the sciatic nerve, in both the control and undernourished rats, there was a large number of fibres of small diameters (0 – 3 μm) during the early postnatal period. Fibre size increased with the increase in age of the animals and the number of fibres of larger diameter increased significantly by the 40th postnatal day (table 2). In the control rats, there was a rapid increase in the population of bigger fibres (3 to 6 μm) with simultaneous reduction in the number of smaller fibres (0 to 3 μm) between 10 and 15 postnatal days. Thereafter, the increase in number of fibres was not significant though at 40 days large number of fibres measuring more than 6 μ were seen. In the undernourished animals the number of small fibres remained high in all the experimental stages when compared with the control. There was some increase in the number of large fibres in the undernourished animals in the 15, 20 and 40 days period but they were significantly less in number than those in the control animals. In the rehabilitated rats, although there was some recovery in the fibre size the fibres of smaller diameter remained significantly high (table 3).

Myelin thickness increased in the control animals with the advancement of age. In the undernourished rats myelin thickness was significantly less than that in the control on all the experimental days (table 4). It increased in rehabilitated rats. Although recovery was near normal in the rats rehabilitated after 10 and 15 days of undernutrition, thickness remained much lower in the animals rehabilitated after 20 days of undernutrition (table 4). No structural alterations like fragmentation of myelin or any significant abnormality in the axons were present in the undernourished rats.

Undernutrition and Nerve Regeneration

In both control and undernourished animals the

Table 2 *Sciatic nerve fibre distribution in control (C) and undernourished rate (U) (The value represent mean±SD)*

Diameter in micron	Days							
	10		15		20		40	
	C	U	C	U	C	U	C	U
0 – 1.5	56.0±10.23	85.67±17.01	33.6±10.407	71.33±7.024	38.0±21 633	80.667±11.015	11.333±2.082	41.25±13.937
1.5 – 3	535.0±24 82	680 33±61.08	303.2±38.648	643.67±64.143	309.33±65.432	471.0±38.118	172.33±38.63	196.5±22.279
3 – 6	409.0±21.307	234.0±77.35	663.2±43.849	285.0±69.462	652.67±60.976	448.33±29.297	488.33±73.962	573.10±35.223
> 6	–	–	–	–	–	–	328.7+77.737	189.00+21.603

Table 3 *Sciatic nerve fibre distribution in control and rehabilitated rats*

Diameter	Days			
	40	10	15	20
	Conrol	Rehabilitated		
0 – 1.5	11.33±2.082	30.33±9.713	30.667±4.041	28.33±6.658
1.5 – 3	172.33±38.631	200.0±12.288	229.0±12.767	240.67±13.75
3 – 6	488.83±73.962	603.33±13.615	519.0±15.621	527.0±41.869
> 6	328.0+77.737	166.67+5.686	221.33+3.054	204.0+42.226

The values represent mean±SD

Table 4 *Myelin thickness (in μ) of the sciatic nerve fibres of the control, undernourished and rehabilitated rats*

Batch	Days			
	10	15	20	40
Control	0.46 ± 0.04	0.65 ± 0.022	0.79 ± 0.05	0.92 ± 0.052
Undernourished	0.32 ± 0.032	0.36 ± 0.023	0.46 ± 0.26	0.68 ± 0.035
Rehabilitated	0.97 ± 0.034	0.90 ± 0.031	0.77 ± 0.021	—

The values represent mean ± SD

changes were somewhat similar on the 3rd and 7th day after injury and were characterised by fragmentation of myelin and axonolysis accompanied by mononuclear cell infiltration. However, on the 7th day, axonal sprouts were also seen and were more in number in the control than in the undernourished group. More distinct differences were seen after 15, 25 and 40 days of injury. While the number of phagocytic cells was markedly reduced in control animals after 15 days and they were absent after 40 days, many of them were still seen after 15 and 25 days in undernourished rats and a few were present even on the 40th day after injury. Myelination was also affected in the undernourished animals. While many thinly myelinated nerve fibres indicating myelin regeneration were noted in the control animals after 15 and 25 days of injury and myelin thickness increased further 40 days after injury, in the undernourished group, myelinated fibres were seen in much less number (table 5) and the thickness of myelin was also much less after 40 days of injury. Difference in the Schwann cell population, as detected by immunohistochemical staining for S-100 protein, was also evident in the two groups. While many Schwann cells were seen on 15, 25 to 40 days after injury in control group, their number was lower at all stages in the undernourished group. The number and thickness of regenerating axons at the crushed site were also significantly more in the control animals. By 40 days the crushed site was populated mostly by well myelinated axons of almost normal size in control animals, while in the undernourished group most of the axons were still markedly thin. Ultrastructurally, increased number of lysosomal bodies, many swollen mitochondria and reduction in the number of microtubules were observed in the regenerating axons of undernourished rats.

Discussion

Undernutrition on the Postnatal Growth of Skeletal Muscle and Nerve

Postnatal growth of skeletal muscle is mostly by increase in size, length and cross sectional area of the individual muscle fibres accompanied by multiplication of sarcolemmal nuclei (Enesco & Puddy 1964, Aharne et al. 1971). Increase in size is more rapid between 15 and 40 days (Gopinath et al. 1983).

In the rats subjected to postnatal undernutrition the diameter of muscle fibres is significantly reduced and the reduction is more pronounced with **the duration of undernutrition** (table 1). Glore and Layman (1987) have shown that in rats subjected to prolonged undernutrition extending upto 120 days after weaning, there is reduction in the weight of skeletal muscles and in the length and cross sectional area of muscle fibres though there is no change in the number. After

Table 5 *Myelinated fibre density (fibres/mm^2) at the crushed site after injury (mean ± SD)*

Group	Days	
	15	40
I	15720 ± 648	17212 ± 755
II	12352 ± 725	14788 ± 642

$^*P < 0.01$ compared with control

rehabilitation, there is some degree of recovery with the increase in the diameter of muscle fibres. However, the recovery is not complete, particularly when the period of undernutrition is prolonged. In case rehabilitation is instituted early, the recovery is very good—the diameter reaching nearly that of the control animals. The diameter remains significantly reduced if the rehabilitation is delayed (table 1). In their study, Glore and Layman (1987) have demonstrated that after prolonged refeeding upto 196 days, although there is significant recovery (reaching upto 84% of cross sectional area of muscle fibres in control animals), the recovery is not complete. It is interesting that in rats subjected to neonatal undernutrition, only reduction in the length and diameter of muscle fibres occur and no other significant structural alteration is noted in the sarcoplasm or in the nuclei, even by EM. However, in animals subjected to severe protein-calorie malnutrition, in addition to reduction in the size of muscle fibres, marked thinning of myofibrils and focal loss of myofilaments have been observed by EM (Roy et al. 1972, Dastur et al. 1979, Oldfors et al. 1983).

In the control rats, there is progressive increase in diameter of nerve fibres (table 2). From the preponderance of fibres of smaller diameter in the early neonatal period, larger diameter fibres become more frequent in the later stage. In neonatal undernutrition number of fibres of smaller diameter is higher than that of normal rat at all postnatal ages in our study. Although increase in fibres of larger diameter does occur in later postnatal age, the number of fibres measuring more than 6μ in diameter remains significantly less in the undernurished rats. Besides the reduction in diameter, reduction in thickness of myelin sheath and in the number of myelin lamellae also occurs in undernourished animals (Gopinath et al. 1983, Hedley White 1973, Sima 1974b, Cornbath & Brown 1988). Reduction in the internodal length has also been observed (Nordbrog 1978). In the rehabilitated animals some recovery in the size of nerve fibres occurs though the number of fibres of smaller diameter (< 6 μm) remains high with significantly lesser number of large fibres ($>$ 6 μm) (table 3). The myelin thickness also remains significantly lower, particularly when rehabilitation to normal diet is delayed for 20 days (table 4). It is to be noted that no other structural alteration is seen in the myelin or axon in undernourished rats. However, in animals subjected to severe protein deficiency, degenerative changes like infolding of myelin sheath, segmental demyelination, reduction of internodal length, axonal shrinkage and excessive accumulation of 10nm filaments and mitochondria within axons have been reported (Roy et al. 1972, Oldfors & Persson 1982, Chopra et al. 1986).

Regeneration of Peripheral Nerve in Neonatal Malnutrition

It is apparent from the present study that regeneration of peripheral nerve after crush injury in neonatal rats is markedly affected by undernutrition. Number of Schwann cells, fibre diameter and myelin thickness were all found to be reduced in the experimental group. Inhibition of Schwann cell proliferation, as indicated by the presence of S-100 protein in their cytoplasm, appears to be due to the direct effect of nutritional deficiency during the period of rapid growth of nerve and this in turn is probably the main reason for reduced myelin formation. The rise of S-100 reactivity has been found to have good correlation with regeneration of rat sciatic nerve (Kato et al. 1983). Reduced axonal growth as seen in the present study may also be responsible for defective myelination, since distension of growing axons may act as a non-specific stimulus for myelination (Friede 1972).

It is possible that regeneration of axons is affected due to reduced protein synthesis in the neurons, thus resulting in deficiency in the formation of axoplasm including neurotubules and neurofilament proteins. Distrubance of normal metabolic activity particularly affecting protein synthesis in neurons has been reported (Griffin et al. 1977 Benay-Schwartz et al. 1979).

The impaired regenerative activity of peripheral nerve after injury may be of great significance in human, especially in undernourished children where neuromuscular apparatus is already compromised (Sachdev et al. 1971, Kumar et al. 1977, Chopra et al. 1986, Lundbrog 1987).

Acknowledgements

The authors acknowledge the support of the WHO for funding the project. They thank the Indian Council of Medical Research and the Indian Journal of Medical Research for the permission to reproduce the tables 1, 2, 3 and 4 from our earlier publication in IJMR (Volume 77, May 1983). They also thank Miss D Tuteja for the secretarial assistance.

References

Aharne W, Ayyar D R, Clarke P A, Walton J B 1971 Muscle fibre size in normal infants, children and adolescents. An autopsy study; *J. Neurol. Sci.* **14** 171-182

Benay-Schwartz M, Giuffrida R M, Deguzman T, Sershen H, Lajtha A 1979 Effect of undernutrition in cerebral protein metabolism; *Exp. Neurol.* **65** 157-168

Chopra J S, Dhand U K, Mehta S, Bakshi V, Rana S, Mehta J 1986 Effect of protein calorie malnutrition on peripheral nerves. A clinical, electrophysiological and histopathological study; *Brain* **109** 307-323

Chowdhury C, Gopinath G, Roy S 1982 Effect of undernutrition on the maturation of Purkinje cells in the rat; *Indian J. Med. Res.* **75** 559-566

Cornbath D R, Brown M J 1988 Influence of malnutrition of developing rat peripheral nerves; *Exp. Neurol.* **99** 403-412

Dastur D K, Daver S M, Manghani D K 1979 Changes in muscle in human malnutrition with an emphasis on the fine structure in protein-calorie malnutrition; in *Progress in Neuropathology* pp 299-318 ed H N Zimmermar (New York: Raven Press)

Dobbing J 1970 Undernutrition and the developing brain. The relevance of animal models to the human problem; *Am. J. Dis. Child.* **120** 411-415

Dobbing J, Sands J 1971 Vulnerability of the developing brain. IX. The effect of nutritional growth retardation on the timing of the brain growth; *Spurt. Biol. Neonate* **19** 363-370

Dyson S E, Jones D G 1976 Undernutrition and developing nervous system. *Prog. Neurobiol.* **7** 171-205

Enesco M, Puddy D 1964 Increase in the number of nuclei and weight in skeletal muscle of rats at various ages; *Am. J. Anat.* **114** 235-244

Friede R L 1972 Control of myelin formation by axon caliber. *J. Comp. Neurol.* **144** 233-252

Gambetti P, Autilio-Gambetti L, Rizzuto N, Shafer B, Pfaff L 1974 Synapses and malnutrition Quantitative ultrastructural study of the rat cerebal cortex; *Exp. Neurol.* **43** 464-473

Glore S R, Layman D 1987 Cellular development of skeletal muscle of rats during recovery from prolonged undernutrition; *J. Nutr.* **117** 1767-1774

Gopinath G, Roy S, Karmarker M G 1981 Effects of undernutrition and rehabilitation on the cerebal cortex in rat; *Indian J. Med. Res.* **74** 866-871

——, ——, —— 1983 Effect of undernutrition and rehabilition on the postnatal growth of skeletal muscle and nerve in the rat; *Indian J. Med. Res.* **77** 702-712

Griffin W S T, Woodward D J, Chanda R 1977 Malnutrition and brain development: cerebal weight, DNA, RNA, protein and histological correlation; *J. Neurochem* **28** 1269-1279

Haltia M, Bertin O, Schucht H, Sourander P 1978 Postnatal differentation and growth of skeletal muscle fibres in normal and undernourished rats; *J. Neurol. Sci.* **36** 25-39

Hedley-White E T 1973 Myelination of rat sciatic nerve. Comparison of undernutrition and cholesterol biosynthesis inhibition. *J. Neuropathol. Exp. Neurol.* **32** 284-302

——, Mauser C S 1971 The effect of undernutrition on myelination of rat sciatic nerve; *Lab. Invest.* **24** 156-161

Kato K, Satoh T 1983 Changes in the concentration of enolase isoenzymes and S-100 protein in degenerating and regenerating rat sciatic nerve; *J. Neurochem.* **40** 1076-1081

Kumar A, Ghai O P, Singh N 1977 Delayed nerve conduction velocities in children with protein calorie malnutrition. *J. Paediatrics* **90** 149-150

Lundborg G 1987 Nerve regeneration and repair. A review; *Acta Orthop. Scand.* **58** 145-169

Mendes C B, Waterlow J C 1958 The effect of low-protein diet, and of refeeding, on the composition of liver and muscle in the weanling rat; *Br. J. Nutr.* **12** 74-88

Nordborg G 1978 The influence of protein-calorie malnutrition on the development of internodal segments. A study of peripheral nerve and spinal roots in rats; *Acta Neuropathol.* **41** 103-108

Oldfors A, Persson M 1982 Peripheral nerve fibre degeneration in protein deprived young rats; *Acta Neuropathol.* **57** 1-6

Oldfors A, Mair MG, Sourander P 1983 Muscle changes in protein—deprived young rats. A morphometrical, histochemical and ultrastructural study; *J. Neurol. Sci.* **59** 291-302

Roy S, Naunihal Singh, Deo M G, Ramalingaswami V 1972 Ultrastructure of skeletal muscle and peripheral nerve in experimental protein deficiency and its correlation with nerve conduction studies; *J. Neurol. Sci.* **17** 399-409

Sachdev K K, Taori G M, Pareira S M 1971 Neuromuscular status in protein-calorie malnutrition: clinical, nerve conduction and electromyographic studies; *Neurology* **21** 801-812

Salas M 1980 Effects of early undernutrition on dendritic spines of cortical pyramidal cells in the rat; *Dev. Neurosci.* **3** 109-117

——, Diaz S, Nieto A 1974 Effects of neonatal food deprivation on cortical spines and dendritic development of the rat; *Brain Res.* **73** 139-144

Sarkar C, Roy S, Gopinath G 1990 Effect of neonatal undernutrition on the brain; *Proc. Indian Natn. Sci. Acad.* **B56** (In Press)

Sima A 1974a Studies on fibre size in developing sciatic nerve and spinal roots in normal, undernourished and rehabilitated rat; *Acta Physiol Scand* **406** 5-12

—— 1974b Relation between the number of myelin lamellae and axon circumference in fibres of ventral and dorsal roots and optic nerve in normal, undernourished and rehabilitated rats. An ultrastructural morphometric study; *Acta Physiol. Scand.* **410** (Suppl) 5-15

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 43-50 (1990)

Effects of Malnutrition and Iron Deficiency on Mental Functions and Study of Possible Mechanisms in Animal Model

K N AGARWAL
Professor & Head, Paediatrics and Coordinator, Nutrition Centre, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005 INDIA

In a study conducted on 25 newborns of undernourished mothers (hemoglobin <8.0 g/dl and serum albumin <2.5 g/dl) during the period 1978-79, it was observed that the placental weight and protein content, and birth weight were significantly reduced. These babies had (i) hypotonia in 72%, (ii) marked hypoexcitability in 56%, and (iii) modification of responses in several reflexes, viz. limp posture, poor recoil of limbs, incomplete Moro's and crossed extensor responses as compared to 23 newborns of well nourished mothers (Bhatia et al. 1979). Of these 19 newborns of undernourished mothers were examined for polygraphic sleep cycle studies (simultaneous recording of EEG, ECOG phasic body activity and respiration). They demonstrated: (i) shortening of sleep cycle; (ii) reduced duration of both active REM (rapid eye movement) and quiet NREM sleep, and a still greater reduction in duration of active REM for babies weighing < 2000 g; (iii) generalised disorganization of sleep, leading to increased duration of intermediate sleep; and (iv) in some, inter- and intrahemispheric asymmetry and abnormal paroxysmal discharges (Bhatia et al. 1980).

Key Words: Malnutrition, Iron deficiency, Mental functions, Animal model.

Introduction

In the available literature the conclusion regarding impact of malnutrition on mental functions is mainly limited to those children who are admitted to the hospital, being severely affected requiring hospital care. In fact, such children constitute less than 5% of poor rural preschool children whereas the study planned by us was to examine children in the community representative of 80% of the child population in countries like India with mild to moderate undernutrition in an endemic form. It has been generally believed that mild and moderate degrees of growth retardation are by and large evidences of adaptation. Stunted children with appropriate weight for height could be considered as adapted and are believed not to be accompanied by functional impairment. The observations for the present study, however, contradict such assumption.

Materials and Methods

Two rural children cohorts (A & B) were selected for repeated observations:

(*A*) 1981 onwards continued: Children from birth to two years of age followed for their physical growth and mental functions.

(*B*) 1983-87: A population of children at school entry point (6-8 years) followed for 3 years.

These children were from the villages which had infant mortality rate of 133‰ and over 26% of births being low birth weight (weight < 2500 g). These villages represented a typical rural demography pattern for 80% of the Indian population. Among pre-school children 13% had severe and 50% had mild to moderate form of protein energy malnutrition. Firstly, the data for the children at school entry point on their nutritional status and mental function, are presented (cohort-B).

Results and Discussion

Intelligence and Cognition in Malnourished School Children

Studies were conducted on 1136 rural children at school entry (6-8 years of age). Among these, 15.8% had weight for age <90% of the NCHS, while the remaining were undernourished (50.8% in grade I, 31.1% in grade II and 2.3% in grade III). In 42.8% height was 90% expected for age considered as normal. The observations on mental functions assessed on the basis of various tests confirmed that: (i) the intelligence (WISC) and cognitive development (Piaget test) are affected in undernourished children and the risk increased with the severity of malnutrition; (ii) the malnutrition affected verbal reasoning, comprehension, perceptual and spatial abilities, fine-motor coordination, short term memory and scholastic achievement, the influence being independent of I.Q. of the child; (iii) children who had stunting (height <90% of the expected for age) were affected more on all functions (Agarwal et al. 1987, 1989); (iv) however, overnight fasting did not influence attention, concentration and short term memory as judged by arithmetic, digit span and short term memory tests (Upadhyay et al. 1988).

Soft Neurological signs and EEG Changes

In a subsample of 208 boys without any neurological deficit seven motor tasks were tested (Agarwal et al. 1989): (i) Finger tapping—repetitive touch movements of thumb and index finger, (ii) Successive finger tapping using thumb as a common finger and successively touching the other four fingers rapidly, (iii) Toe—tapping-distant tapping keeping heel on floor, (iv) Heel-toe tapping—movements conducted in rocking fashion, each time either heel or toe being on the floor, (v) Hand patting—clapping with minimal distance each time being 9 cm, (vi) Arm pronation supination—alternating such movements keeping elbow flexed at 90^0, (vii) Hand flexion-extention—conducted at elbow joints keeping palm fisted—20 movements of each motor task finger tapping, successive finger tapping, toe tapping, heel toe tapping and hand flexion extension were timed. It was observed that 4.2% children having normal nutrition showed soft neurological signs. However, these were present in 48.3% of grade I and 87.3% of grade II undernourished children. For successive finger tapping, toe tapping, heel-toe tapping, hand patting and arm pronation supination there was significant influence of malnutrition on both sides, i.e. for finger tapping on right and hand flexion-extension of left. In 16 children with soft neurological signs EEG findings showed:

(a) Frontal lobe slow and sharp wave pattern in 10 out of 16 children accompanied with soft neurological signs elicited on the following three motor tasks-successive finger tapping, heel toe tapping and arm pronation supination.

(b) Temporal lobe was involved in three children who had poor performance on successive finger tapping.

(c) Parietal lobe involvement in two children was accompanied with poor heel toe tapping, arm pronation supination, while one child also showed poor successive finger tapping.

Out of 16 EEG reports, one was normal, although there was presence of poor performance on successive finger tapping and arm pronation supination. It seems these simple motor tasks can be used to identify children who have poor fine motor coordination as a screening method in the field. On the basis of these findings, the motor and mental functional deficits may be summarized as follows:

	Malnutrition	
	Mild	Moderate & severe
Soft neurological signs	Successive finger tapping, heel toe tapping, toe tapping, arm pronation supination	Successive finger tapping heel toe tapping, toe tapping, arm pronation supination, hand patting, hand flexion-extension
Mental functions affected	Attention, concentration	Verbal ability, perceptual function, memory.

Having found changes in 6-8 yr school children (8-10 yr for soft neurological signs and EEG), we correlated our findings in children observed from birth to 6 yr of age. It was noted that on intelligence test (Binet Kulsrestha) language, comprehension, reasoning and immediate memory were significantly affected. The visual perception was also affected but to a lesser extent.

In 4 - 6 yr of age group, finger tapping, toe tapping, hand patting, arm pronation supination and hand flexion extension were affected. These children took significantly more time in stringing 50 beads (mean 18.16±2.51 sec) as against the control (13.78±3.86 sec). Similarly, undernourished children placed less number of pegs in 60 sec (15.±2.3) as compared to their wellnourished counterparts (19±4.4). Further, out of 13 children, 11 had an abnormal EEG (sharp and slow wave abnormality, temporal lobe - 4; temporoparietal-3; parietal - 2; frontal-temporal - 2).

The findings on motor coordination, language and comprehension, visual perception, immediate memory, reasoning and concept formation are summarized for both the cohorts (A & B) in table 1 (Agarwal et al. 1987, 1989, Agarwal 1989 Upadhyay 1988, 1989a & b).

Impact of Mid-day Nutrition Supplementation

Nutrition supplementation could be regularly given to 146 school children for two years, this intervention showed marginal increment in I.Q. scores. Incognitive tasks improvement was observed for conservation of liquid. The scores on arithmatic achievement test also showed improvement (Agarwal et al. 1989)

Iron Deficiency—mental Functions

In India, besides protein energy malnutrition, iron deficiency with or without anemia is also a common form of nutritional deficiency. It was found that children with iron deficiency anemia had poor mental functions, i.e. attention, concentration and scholastic achievement (particularly arithematic). However, while their nutritional status was controlled, it was observed that iron deficiency alone caused poor attention and concentration (Agarwal et al. 1989)

Study of Possible Mechanisms for Altered mental functions in Rat Brain Models

In view of the above human data, it was planned to study the brain in experimental model, particularly for neurotransmitter enzymes in rats fed on cereal and/or pulse diets or on iron deficient diet as these are the diets commonly consumed by the communities in India.

(a) *Nutritional deprivation: Effects on brain*

In India, foods especially cereals and pulses deficient in one or more amino acids (low quality protein), are the major sources of protein, i.e. wheat, rice, corn, pulse, vegetables, etc. in the vulnerable periods of pregnancy, lactation and early childhood (Agarwal & Prasad 1981). In fetuses of rat mothers maintained on wheat or Bengal gram diets isonitrogenous with 10% casein diet body weights were unchanged while brain weight was reduced (Prasad et al. 1979 and Agarwal et al. 1981). It was further demonstrated that such diets result in lower protein, DNA and RNA content in fetal brains and also a reduced RNA/DNA ratio, indicating decreased protein synthesis. These effects could be countered by supplementing the diets with deficient amino acids. However, the above parameters remained unaltered when isonitrogenous diets were fed to 6-week-old rats. The studies on glutamic acid and GABA and their synthesising and degrading enzymes on such diets demonstrated that the activities of glutamine synthetase, glutamine transferase, glutaminase I and II and glutamate decarboxylase and the concentrations of free amino acids, glutamine, alanine and GABA also decreased Prasad et al. 1979. (Agarwal & Prasad 1980, 1981, Prasad & Agarwal 1980, Agarwal et al. 1980, 1981).

These studies do create a doubt that vegetarian communities subsisting on imbalanced protein diets may be exposed to greater risk of retardation of brain growth during nutritional deprivation. These findings in animals suggest that Indian population suffering from endemic undernutrition, especially during pregnancy and early childhood is likely to be affected in mental functions and possibly in fine motor coordination (Agarwal et al. 1987, 1989)

In human fetal brain proportionate reduction in weight which was more marked for cerebellum and medulla oblongata, was observed. The brain-body

Table 1 *Effect of nutrition on behaviour development and intelligence in children living in endemic undernutrition*

Abilities	Age				Comments
	0-3 years		6-8 years		
	Tests	Results	Tests	Results	
Motor coordination	Adaptive behavior scale of Gesell development schedules	Significantly affected	Bender-Gestalt test	No effect when other variables controlled	More affected in early age improved with age
	Binet-Kulshreshtha intelligence test (at 3 yrs)	Significantly affected	Assessment on maze, coding and block desing subtests of Malin's scale	Weak association with nutrition. It was less as compared to verbal abilities	
	Soft neurological sings and EEG (4-6 yr)	Significantly affected	Soft neurological signs and EEG (8-10 yr)	Significantly affected	
Language and comprehension	Gesell developmental schedule	Significant effect from 18 months onward	Assessment on information and comprehension subtests of Malin scale, Piaget's conservation tasks	Significantly affected	Effect from 1 yr and continued upto 8 yr
	Binet-Kulshreshtha intelligence test (at 3 yr)	Significantly affected			
Visual perception	Binet-Kulshreshtha intelligence test	Was affected to a lesser extent	Bender-Gestalt test, performance subtests of Malin's scale	Significant but weak association with nutrition. It was of a lower magnitude compared to verbal abilities	Minimal effect of undernutrition
Immediate memory	Binet-Kulshreshtha intelligence test (at 3 years)	Significantly affected	Digit span of Malin's scale Memory test	Significantly affected	Continued from early age till school period
Reasoning	Binet-Kulshreshtha intelligence test (as 3 years	Significantly affected	Piaget's conservation tasks	Significantly affected	Continued from early age till school period
Concept formation	Block-sort test (at 3 years)	Significant effect of nutrition. In multiple regression analysis effect of home environment was significant- No effect of nutrition in children matched for IQ	Similarities subtests of Malin's scale	No effect when other variables were controlled	No effect of undernutrition in either age group

Table 2 *Effect of latent iron deficiency on GABA, glutamate and 5-HT metabolism in rat brain*

Parameters	Post-weaning rats	After rehabilitation	Pre-weaning rats (whose mothers were iron deficient during pregnancy and lactation)	After rehabilitation
Glutamate	Decreased	Rev		
GABA	Decrased	Rev		
Tryptophan	Decreased	Partial Rev		
5-HT	Decreased	Irr		
5-HIAA	Decreased	Irr		
Tyrosine	Decreased	Irr		
Dopamine	No change			
Nerepinephrine	No change			
Glutamate dehydrogenase (GDH)	Decreased	Rev	Decreased	Irr
Glutamate decarboxylase (GAD)	Decreased	Rev	Decreased	Irr
GABA-transaminase (GABA-T)	Decreased	Rev	Decreased	Irr
NAD±isocitrate dehydrogenase	Decreased	Rev	Decreased	Irr
Monoamineoxidase (MAO)	No change			
Aldehyde dehydrogenase	No change			
Tyrosine Amino transferase (TAT)	Increased	Rev		

Rev, Reversible; *Irr*, Irreversible

Table 3 *Effect of feeding iron deficient diet (18-20 mg iron/kg) to postweaning rats for 8 weeks on various trace elements*

Different brain regions	Various trace elements						
	Fe	Zn	Cu	Ca	Cd	Pb	Mn
Corpus striatum	32%		16%	22%			
Mid brain	21%	16%^{R}					
Hypothalamus	19%			17%^{R}			
Cerebellum	18%				19%		18%
Cerebral cortex	17%		18%				
Hippocampus	15%	19%^{R}				17%	
Medulla oblongata	NC						

R, Reversible; *NC*, No change; Decreased; Increased
All other changes are irreversible after rehabilitation

weight ratios demonstrate that the body weight is affected more in the intrauterine undernutrition as compared to brain weight. Further, it was observed that cerebellum and brain stem weights are proportionately more affected than the cerebrum (Agarwal 1984).

Latent iron deficiency: Effect on brain (table 2 & 3) dam & Offspring: A model was developed in rat by feeding iron deficient diet (35 mg iron/kg) prior to pregnancy and during lactation. These mothers did not develop any change in brain enzyme activities of GABA shunt except reduction in hepatic non-heme iron content which was progressive. Fetal weight, brain-weight, protein, DNA and RNA showed no change when examined on 20th day of intrauterine life to 21 day of postnatal age (Taneja et al. 1989)

Post-weaning rat brain: The groups of rats fed on 35 mg and 18—20 mg iron/kg diet showed reduction in aminobutyric acid and glutamic acid contents in the brain. The activities of GABA shunt enzymes, glutamate dehydrogenase (GDH), glutamate decarboxylase (GAD), GABA-transaminase (GABA-T) and of the NAD=linked isocitric dehydrogenase (ICDH), were reduced. The brain tryptophan, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid contents were reduced (Taneja et al. 1986 and Shukla et al. 1989).

Pre-weaning rat brain: The latent iron deficiency during gestation and lactation showed reduction in brain enzyme activities of GDH, GAD, GABA-T and NAD$\underline{+}$linked ICDH in 14 and 21-day-old pups (Shukla et al. 1981 and Taneja et al 1986, 1989;). Rehabilitation on 390 mg iron/kg diet showed that: (i) in post a weaning rats, altered GABA and glutamate metabolism reversed, inspite of the fact that the reduced iron content of the brain did not improve. However, 5-HT metabolism (Tryptophan, 5-HT, 5-HIAA and tyrosine reduction) alterations did not improve; and (ii) in pre-weaning rats, reduced brain enzyme activities of GDH, GAD, GABA-T and NAD$\underline{+}$ linked ICDH, remained reversible.

Brain metal levels: Iron content was reduced in all regions, except medulla oblongata (table 3). The maximum reduction in iron content being in corpus striatum which remained irreversible on rehabilitation. Zinc Content increased in mid-brain and hippocampus, which normalised on rehabilitation.

Relationship with studies on feto-placental unit—

Maternal anemia: The maternal iron deficiency anemia affects placental and cord serum iron and ferritin levels (Singla et al. 1979, Agarwal et al. 1983). There is reduction in placental weight, volume, surface area, number of cotyledons (Khanna et al. 1979), cell number (Marwah et al. 1979) and reduced SDH enzyme activity in maternal anemia. The birth weight was also significantly reduced with the degree of anemia. There was low hepatic iron content in babies born of these mothers (Singla et al. 1985). These newborns had poor Apgar scores (Agarwal 1984)

Maternal Undernutrition

Similarly earlier studies by Agarwal and associates (1976, 1977a,b, 1984) have demonstrated alterations in placental weight, volume, protein, transaminases, in histological changes and higher prevalence of low birth weight babies born of undernourished mothers.

Conclusions

The human as well as animal studies (presented above) revealed that intrauterine malnutrition/iron deficiency are likely to affect mental functions. These studies also indicate that some of these deficits may be irreversible. Although these deficiences may not result in frank cases of mental retardation, in intellectual as well as in cognitive development such children are inferior. Furthermore, a great number of them will grow with poor fine motor coordination which may make them unfit for precision and high technical jobs in later life. It appears that the critical periods of human brain growth (intrauterine and the first year of life) is very important in terms of nutritional support, instead of such supplementation in the ICDS system (after 2½ years of age) or through mid-day meal school programme. We have also demonstrated in animal experiments, alterations induced by nutritional and iron deficiencies in GABA and glutamate metabolism, in 5-HT metabolism, and in metal contents in various parts of the brain. These changes are irreversible if the deficiency occurs in intrauterine life or during weaning period. It may be concluded that similar changes also take place in human brain as well, and are responsible for permanent functional deficits observed.

References

Agarwal K N and Prasad C 1980 Effect of wheat and Bengal gram diets on the brain glutamate metabolism in post-weanling rats; *J. Nutr.* **110:** 2166

Agarwal D K, Upadhyay S K, Tripathi A M and Agarwal K N 1987 Nutritional status, physical work capacity and mental function in school children; in *Nutrition Foundation of India*, Scientific Report **6:**pp 1-86

Agarwal D K, Upadhyay S K and Agarwal KN 1989 Influence of malnutrition on cognitive development assessed by Piagetian Tasks; *Acta Paediatr Scand* **78:** 115-122

Agarwal D K, Agarwal K N and Upadhyay S K 1989 Effect of mid-day meal programme on physical growth and mental function; *Ind. J Med Res* **89:** 163-174

—— and —— 1981 Effect of wheat and Bengal gram diets on glutamic acid metabolism in rat brain; *Indian J. Med. Res.* **73** 350-356

——, Das D, Agarwal D K, Upadhyay S K and Mishra S 1989 Soft neurological signs and EEG pattern in rural malnourished children; *Acta Paediatr Scand* **78:** 873-

——, —— and Taneja V 1981 Effect of poor quality protein diets on foetal brain growth of rat; *Comp. Physiol. Ecol.* **6** 103-107

Agarwal K N, Taneja V and Prasad C 1980 Effect of low quality dietary proteins on brain protein and enzymes of glutamic acid metabolism in rats; *Indian J. Med. Res.* **71:** 758-763

Agarwal D K, Upadhyay S K, Agarwal K N, Singh R D and Tripathi A M 1989 Anemia and mental functions in rural primary school children. *Ann Trop Paediatr* **9:** 194-198.

——, —— and —— 1981 Protein deprivation and the brain: Effect on enzymes and free amino acids related to glutamate metabolism in rats; *Nutr. Metab* **25:** 228-233

Agarwal K N 1984 Effect of maternal undernutrition on fetal and postnatal brain growth. *INSA Status Report Series* 1. pp 78-83 Ed P N Tandon & G Gopinath.

—— 1984b The effect of maternal iron deficiency on the placenta and foetus. *Adv. Int. MCH IV. pp. 26-35* (Ed. Jelliffe and Jelliffe; Oxford Univ Press)

Agarwal R M D, Tripathi A M and Agarwal K N 1983 Cord blood haemoglobin, iron and ferritin status in maternal anemia. *Acta Paediatr. Scand.* **72** 545-548

Bhatia V P, Katiyar G P and Agarwal K N 1979 Effect of intrauterine nutritional deprivation on neuromotor behaviour of the newborn; *Acta Paediatr. Scand.* **68:** 561-566

Bhatia V P, Katiyar G P, Agarwal K N, Das T K and Dey P K 1980 Sleep cycle studies in babies of undernourished mothers; *Arch. Dis. Child* **55** 134-138

Khanna S, Chand S, Singla P N and Agarwal K N 1979 Morphological study of placenta in pregnancy anemia; *Indian J. Pathol. Microbiol.* **22** 7-12

Marwah P, Singla P N, Krishna M and Agarwal K N 1979 Effect of pregnancy anemia on cellular growth in the human placenta; *Acta Paediatr. Scand.* **68** 899-901

Prasad C, Devi R and Agarwal K N 1979 Effects of dietary proteins on fetal brain protein and glutamic acid metabolism in rat. *J. Neurochem.* **32** 1309-1314

Prasad C and Agarwal K N 1980 Intrauterine malnutrition and the brain: Effects on enzymes and free amino acids related to glutamate metabolism; *J. Neurochem.* **34**.1270-1273

Prasad C, Mehta S and Agarwal K N 1979 Brain free amino acids of glutamic acid system in growing rats fed wheat and Bengal gram protein diets; *Indian J. Exp. Biol.* **17** 516-517

Singla P N, Chand S and Agarwal K N 1979 Cord serum and placental tissue iron status in maternal hypoferremia; *Am. J. Clin. Nutr.* **32** 1462-1465

Singla P N, Shankar R, Ahuja R and Agarwal K N 1987 Placental oxidative enzymes in pregnancy anemia; *Indian J. Med. Res.* **85:** 176-180

Singla P N, Gupta V K and Agarwal K N 1985 Storage iron in human fetal organs; *Acta Paediatr. Scand.* **74** 701-706

Shukla A, Agarwal K N, Chansuria J P N and Taneja V 1989 Effect of latent iron deficiency on 5-hydroxytryptamine metabolism in rat brain; *J Neurochem* **52:** 730-735

——, —— and Shukla G S 1989 Latent iron deficiency alters gamma-aminobutyric acid and glutamate metabolism in rat brain; *Experientia* **45** 343-345

Shukla A, Agarwal K N and Shukla G S 1989 Effect of latent iron deficiency on metal levels of rat brain regions; *Biol Trace Element Res.* **22:** 141-

Taneja V, Mishra K P and Agarwal K N 1989 Effect of maternal iron deficiency in rat on the fetal and postnatal brain GABA shunt; *Indian J. Exp. Biol.* April 1990.

Taneja V, Mishra K P and Agarwal K N 1986 Effect of early iron deficiency in rat on the gamma-aminobutyric acid shunt in brain; *J. Neurochem* **46** 1670-1674

Upadhyay S K, Agarwal D K, Agarwal K N, Srivastava K B and Adhikari G S 1988 Brief fasting and cognitive functions in rural school children; *Indian Pediatr.* **25** 288-289

Upadhyay S K, Agarwal D K and Agarwal K N 1989a Influence of malnutrition on social maturity, visual motor coordination and memory in rural school children; *Indian J. Med. Res.* **90:** 320-327.

Upadhyay S K, Agarwal D K and Agarwal K N 1989b The influence of malnutrition on intellectual development. *Ind. J. Med. Res.* **90:** 430-441

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 51-58 (1990)

Neural Transplantation in Mammals: Our Experience

P N TANDON, GOMATHY GOPINATH, A K MAHAPATRA and A K SHETTY
National Facility for Neural Transplantation, All India Institute of Medical Sciences, New Delhi 110 029 (INDIA)

Neural tissue transplantation was carried out at various sites in rodents and rhesus monkeys using different regions of the fetal brain to establish the technique. Whereas there was 80 – 85% success in rodents, the success rate in monkeys was approximately 20%, that too when the donor embryonic tissue was cryo-preserved for few days prior to transplantation. The significance of this observation is not clear. Further, fetal substantia nigra and adult adrenal medulla were transplanted in the anterior chamber of the eye, the lateral ventricle and the striatum of adult rats for long term morphological studies. Most available studies have been restricted to 3 to 7 months and detailed morphological investigations are lacking. These studies showed that, though nigral cells differentiated to express phenotypic features, there was a gradual "ageing or degenerative" changes observed in transplants of more than six months. A peculiar feature, not seen in the normal substantia nigra was frequent occurrence of intimately opposed neurons. Transplanted adrenal medullary cells did not show any neuronal differentiation and they degenerated at a faster rate.

These observations reveal that the transplanted aminergic cells survive in the host's brain for limited duration and manifest several morphological abnormalities. The neurobiological significance of these findings needs to be explored further. Moreover, in nonhuman primates survival rate of the transplant is poor. These factors need to be taken into consideration before using this procedure for treatment of human diseases.

Key Words: Neural Transplant, Ageing of Transplants, Aminergic Cells in Transplant

Introduction

Nearly three years ago Department of Science & Technology, Government of India, established a National Facility for Neural Transplantation to study neurobiological and behavioural consequences of neural transplants in mammals. A multidisciplinary group working in this Unit has standardized the technique of fetal neural transplantation in rats and monkeys. A variety of sites, e.g. different parts of the brain parenchyma, lateral ventricle and the anterior chamber of the eye, have been utilized in the hosts for transplantation of fetal tissue obtained from different regions of the brain. Besides, autografts and allografts of adrenal medulla have also been similarly transplanted. These studies have interesting implications both from the standpoint of basic neurobiology and also for ultimate use of this technique for therapy of neurological disorders in man.

Material and Method

Initially a variety of sites in the host brain were used to transplant donor tissue from different regions of the developing brain of 17-18 days old rat fetuses. The technique used was one standardized by Das et al. (1979). Successful transplantation could be achieved in 80-85% cases. Having standardized the technique, it was decided to concentration transplantation of dopaminergic neurons from fetal substantia nigra and adult adrenal medulla.

Simultaneously, work was started on rhesus monkeys to ascertain if the results so consistantly obtained by workers all over the world in rodents could be duplicated in sub-human primates. As a prelude to this, monkey fetuses of varying periods of gestation, (50-90 days) were studied to determine the stage of neurogenesis most suitable for purposes of transplantation in the light of experience obtained from rodents (Das et al. 1980 and Gopinath et al. 1987). It is now generally recognised that the donor tissue should have enough growth potential to multiply, differentiate and mature so that it can integrate with the host brain. Mixture of dividing neuroepithelial cells and neuroblasts without mesenchymal contamination is ideally suited for this purpose. Host monkeys were treated with MPTP to produce a model of Parkinsonism (intramuscular injection for bilateral effect and intracarotid for hemiparkinsonism). These monkeys were then transplanted with fetal substantia nigra in their striatum unilaterally.

Both groups of animals were sacrificed at varying intervals and submitted to detailed morphological studies utilizing the following techniques:

(i) Nissel stain for cytoarchitecture
(ii) Electronmicroscopic studies for cytological details
(iii) Golgi stain for details of individual neurons
(iv) Fluorescence for catacholamines
(v) HRP for neuronal tracing and connectivity
(vi) Immunocytochemical studies by gold labelling
(vii) Quantitative morphometry

While several other studies, e.g. transplantation following kainic acid lesions in preoptic and caudate region or transplantation of fetal neocortex in anterior chamber of eye and tectal region have been carried out in rats, the present communication deals with our experience with dopaminergic neurons only. The same applies to our studies in monkeys. Table 1 summarises the sites and nature of donor tissue utilized by us. Details of study in various groups will be published separately. Only a brief summary is being provided here.

Table 1 *Neocortical grafts in rat (A) and Substantia nigral grafts in intact adult rats (B)*

Host site	Total transplants	Successful transplants
Cerebellum	45	25
Caudate	31	20
Lateral ventricle	15	8
Tectum (New born host)	20	16
Intraocular	84	78
Intraventricular	62	53
Intrastriatal	55	46

Results

Rats

Neocortex, substantia nigra, and adrenal medullary transplants in the anterior chamber of the eye, lateral ventricle or the striatum could be successfully established in 70 to 85% adult host rats. These were followed for a period until 1 year after transplantation. To the best of our knowledge such detailed morphological studies, for such long periods are not available in the literature.

As already demonstrated by a host of workers, neural tissue survived, grew, developed organotypic maturation, sent out processes, established synaptic connections and produced appropriate neurotransmitter. While majority of cells remained in clusters, some at the approptiate sites showed migration. Axonal transport could also be demonstrated with the help of HRP labeling. The surrounding brain sent out processes towards the graft and probably into it. It must be mentioned that while all these features were observed in varying degrees in fetal substantia nigra transplants, irrespective of the sites of transplanatation, the anterior chamber of the eye, lateral ventricle or the striatum, the adrenal medullarly transplants did not transform into neurons but retained the characteristics of aminergic cells. This was obviously exciting and held great promise for therapeutic application of the technique.

However, a more critical study of the transplants revealed a number of features which appear to have important implications, if its integration with the host with development of appropriate circuitry and long term survival of the graft are a pre-requisite for recovery of lost functions. Some of these features are summarized below:

i) While the transplanted neurons developed processes, at times indeed very impressive, these seldom extended more than 2-3mm into the host tissue.

ii) These processes manifested some branching but seldom the degree of arborizations seen in normal neurons (figures 1, 2).

iii) The processes, occasionally seen to be growing from the host parenchyma into the transplant, could not definitely be demonstrated to develop synapses with the neurons of the transplant.

iv) Most of the synapses observed were between the neurons of the transplant itself (figure 3).

v) Close approximation of two neurons, not seen in normal substantia nigra, was a common feature in the transplants and gap junctions could be seen between neurons without any associated vesicles. The significance of this observation is not clear (figure 3).

vi) Even more important than the above observations, are the changes observed in these neurons with passage of time. From 60th day of transplantation a number of neuronal soma and a few dendrites started showing clear spaces in the cytoplasam (figure 5). Frequency of such neurons increased as the age of the transplant advanced. As time progressed, one could observe shunken neurons with hyperchromatic nuclei. Thus, the proportion of such neurons to normal neurons was at 1:3 180 days and 1:2 at 360 days.

vii) Similarly there was increasing amount of lysosomal bodies with increasing age of the transplant. This may be indicative of early ageing in these neurons.

It may be emphasized that such changes were observed in transplants at all the three sites, e.g. anterior chamber of the eye, the ventricle and the striatum.

viii) Besides these morphological changes, very likely indicative of degeneration, there was a concurrent decrease in the total number of neurons and fluorescing neurons in the transplant.

ix) Similarly features suggestive of progressive degeneration were observed in the transplanted aminergic cells from adrenal medulla also. (figure 6).

Rhesus Monkeys

Successful transplant in monkeys could be achieved only in 20% of cases, as opposed to 80-85% in rats. It is interesting to note that the fetal tissue used for transplantation in the first two successful transplants in monkeys was preserved in culture medium for 4 days before injecting in the host while all transplants using fresh tissue failed. Following demonstration of successful transplants in these monkeys (figure 7), experimental model of Parkinsonism using parenteral or unilateral intracarotid MPTP injection, was established. Once the syndrome was stabilized, fetal (50 days gestation period) substantia nigra was transplanted in the caudate. Two monkeys (one with generalized and other with hemiparkinsonism) survived long enough for study. There was considerable improvement in tremor, rigidity and akinesia in 2 weeks time in one, even though the movements of the limbs were still slow and clumpsy. This animal was sacrificed 100 days after transplantation, and showed diffuse aminergic fluorescence at the site of transplant.

Discussion

These results confirm, what has already been well established that the fetal neural transplants into the brain of adult hosts, with or without pre-existing lesion, survive, grow, develop organotypic maturation, produce appropriate neurotransmitter and result in varying degree of recovery of lost function. In the present study we have primarily concentrated on morphological aspects using a battery of techniques, light and electonmicroscopy, Golgi, HRP labelling, immunohistochemistry and immunofluorescence. While on the face of it, it appears that in a large measure these investigations are confirmatory in nature, a more careful review of our observations reveal some interesting findings summarized above. These have important implications both from basic neurobiological standpoint as also for the clinical application of the technique.

It is obvious from the present study that while successful transplantation is a rule rather than an exception in rodents, the same is not true for rhesus monkeys. This is not just our experience but others have the same high failure rate. The reason for this failure is not obvious. Though routine studies failed to show significant lymphocytic infiltration , more detailed studies using specific antibodies will be necessary to determine if this is due to immunological rejection. It may be mentioned that in lesser evolved sub-human primates like African green monkeys, cynomolgus monkeys, squirrel monkeys or bonnet monkeys the failure rate is not as high. At the time of this presentation only a few reports of successful transplantation in rhesus monkeys are available (Bakay et al. 1987, Morihisa et al. 1987). Furthermore, detailed morphological studies are still lacking which makes it difficult to evaluate, if the behavioural recovery was really due to the establishment of neuronal circuitry between the host and the transplant.

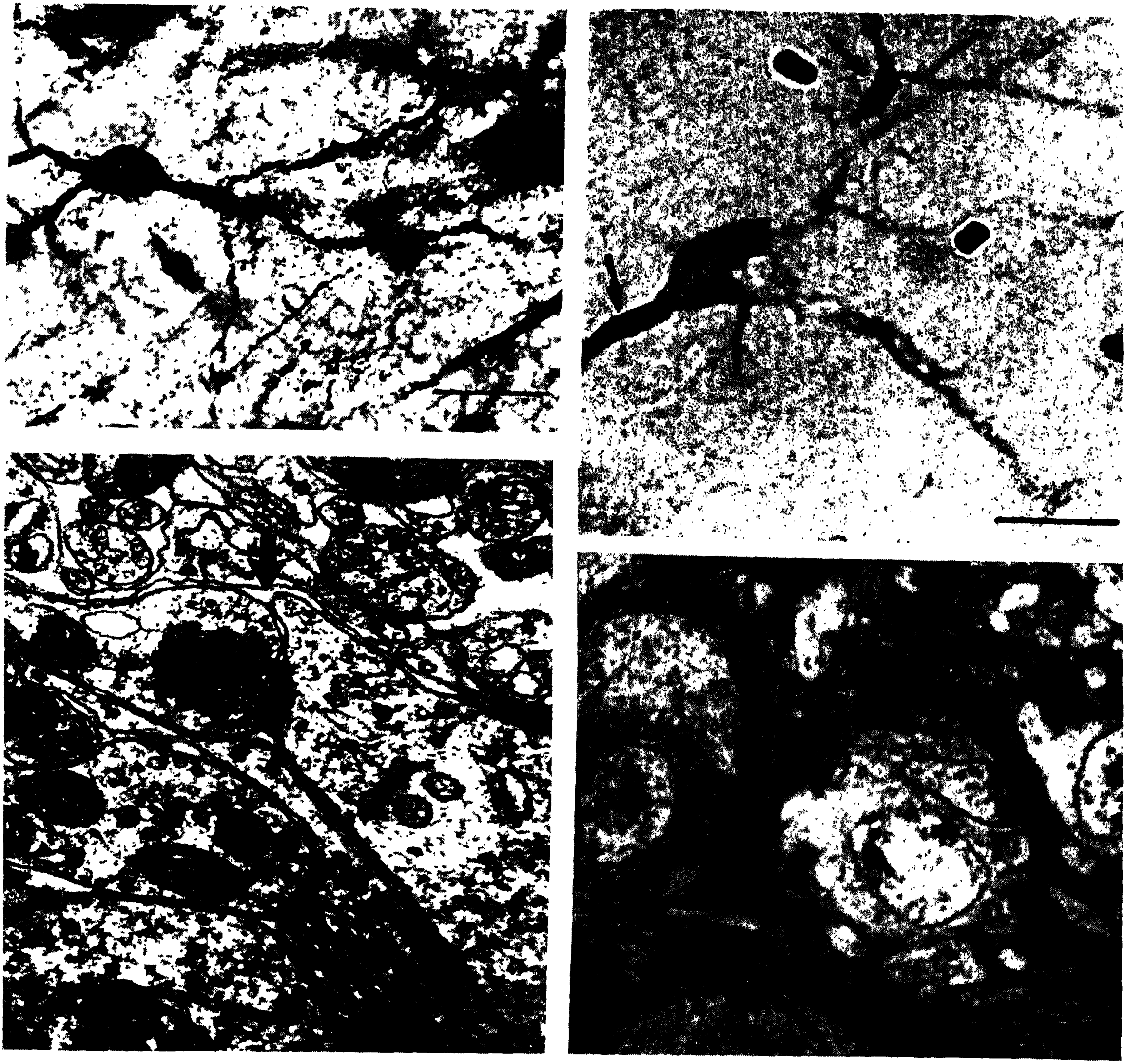
Another intriguing feature regarding transplantation in monkeys was the success obtained with fetal tissue preserved for 4 days in culture media as opposed to the high failure rate of fresh transplants. The presentation by Victorov and Lyjin in this symposium is of special relevance for our observation. This line of investigation need to be persued further, not only to elucidate the biological basis of this observation, but also for its relevance as and when fetal neural transplantation is adopted for clinical use. Needless to say many more animals needs to be studied prior to any definite conclusion.

In respect of the transplantation of dopaminergic tissue from substantia nigra and adrenal medulla in rats, the present study raises some important questions. Firstly while the transplanted cells acquire organotypic maturation, the morphological features are not always completely identical to the normal cells. The significance of frequent observation of close approximation of two neurons, the existence of gap junctions without any associated vesicles is still not clear. Yet these observations must be kept in mind. Furthermore the transplanted nigral enlarge cells do mature into neurons, glial cells and even myelinated fibres, however, the Golgi studies of the neurons show that neither the morphology of dendrites nor their arborizations are similar to the normal neurons of substantia nigra. The neuronal processes grow to a varying extent but seldom more than a millimeter or two. Most of the time these end up in the transplant itself. The synapses observed are between the neurons of the transplant. Thus, at best the neuronal circuitry is restricted locally. Once again the biological implications of this phenomenon have not yet been elucidated. There is no doubt that these neurons produce dopamine but the manner in which this locally produced dopamine helps to ameliorate the clinical symptoms is not clear.

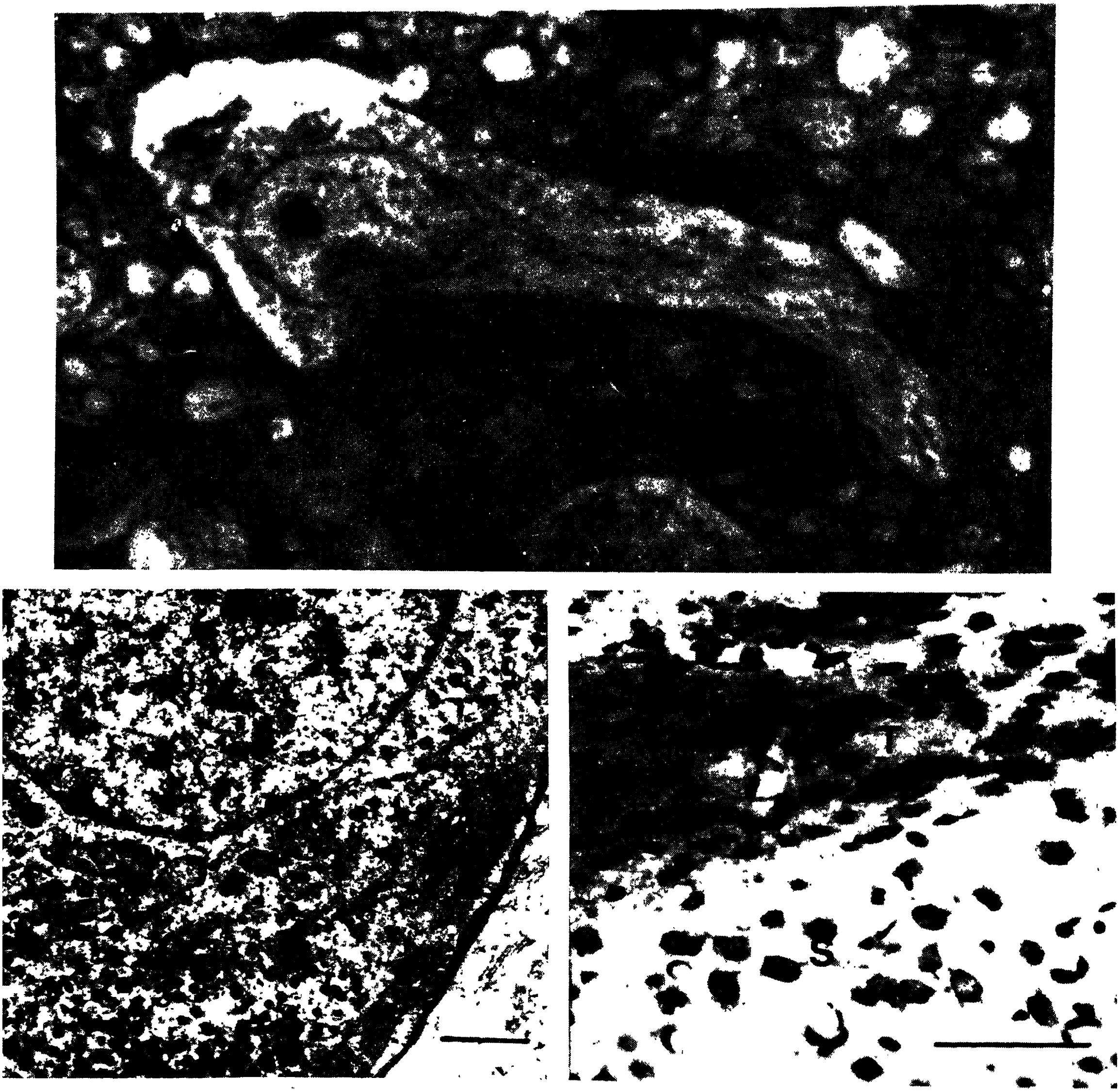
One of the important observations of the present study, so far not commented upon in the published literature, is in respect of progressively increasing number of "degnerating or ageing" neurons observed in animals surviving for more than six months. This does not appear to be an immunological rejection but a true degeneration. It is important to understand the underlying pathogenetic mechanism for this degeneration by further investigations. Needless to say this is of vital importance for its clinical implications.

With regards to the adrenal medullary transplants our observations confirm the earlier findings. However, it may be stated that unlike Olson (1970) and Olson et al. (1980), we did not observe any neuronal transformation of the aminergic cells of adrenal medulla even in the striatum. With increasing duration of transplant the density of aminergic fluorescence diminished. This may be indicative of fall out of the transplanted cells with passage of time.

A few words with regards to clinical implications of our observations in the light of published reports on adrenal medullary transplants in patients of Parkinson's disease would be in place. The immediate improvement in the clinical features of these patients reported by Madrazo et al. (1987) following transplantation in the caudate nucleus obviously cannot be due to growth, integration or development of even local circuitry. One of the possibility is local release of dopamine by the transplanted cells undergoing "lysis". There could be some other "neurotrophic" substance made available by the transplanted cells which may "stimulate" formation or release of dopamine by the host's intact cells. If this be so, one cannot explain the longterm good results claimed by the neurosurgeons atleast in some



Figures 1-4, **1**, A Golgi-stained neuron from the nigral transplant in the striatum of adult rat. Bar=40μ; **2**, A tyrosine hydroxylase positive dopaminergic nigral neuron in the ventricular transplant of 8 months duration. Thickening of the dendrites is seen as part of the ageing process (arrow). (Bar=20μ); **3**, Electronmicrograph showing intrinsic synapse in the intrastriatal transplant grown from fetal substantia nigra (arrow) (Bar=0.5μ); **4**, Photomicrograph of a semithin section of a transplant (introcular) to show myelinated fibres and double neurons. (Bar=10μ) Toluidine blue stain;



Figures 5-7, **5**, A neuron showing clear space in the cytoplasm after 6 month of transplantation (arrow). (Bar = 5μ) Semithin section stained with toluidine blue, **6**, Electron micrograph of a chromaffin cell in the medullary autograft showing degenerative changes of the aminergic granules & organells (Bar = 1μ); **7**, Photomicrograph of a 4 month old transplant (T) grown from fetal neocortex in the striatum (s) of rhesus monkey. (Bar = 50μ) Cresyl violet stain.

cases. It may thus be that development of neuronal circuitry is not a pre-requisite for the beneficial effect of the transplant.

It will be obvious from this presentation that while transplantation of the embryonic neural tissue in adult host of any species is a reality today and not science fiction, there are many remaining questions unanswered before we could safely recommend the procedure for treatment of human disease. It would not be wrong to state that from the rat to man is a long way, but the results in rats (and now in monkeys) are tentalizing enough to prompt intensive multidisciplinary investigations, not only morphological as presented here, but biochemical, immunological and behavioural with a sense of urgency. Even if a fraction of the success already achieved in animal models can be transplanted in human situation an immense amount of suffering can be mitigated.

References

Bakay Rae, Barrow D L, Fiandaca M S, Iuvone P M, Schiff A, Collins D C 1987 Biochemical and behavioural correction of MPTP Parkinson—like syndrome by fetal cell transplantation; *Ann. Ny. Acad. Sci.* **495** 623-641

Das G D, Hallas B H, Das K G 1979 Transplantation of neural tissues in the brains of laboratory animals: Technical details and comments; *Experientia* **35** 143-153

——, —— and —— 1980. Transplantation of brain tissue in the brain of rat. 1. Growth characteristics of neocortical transplants from embryos of different ages; *Am. J Anat.* **158** 135-145

Gopinath G, Mahapatra A K, Nayar U, Tandon P N 1987 Transplantation of the embryonic neocortex in the caudate putamen of adult rat; *Indian J. Med. Res.* **86** 246-252

Madrazo I, Drucker-Colin R, Diaz V 1987 Open microsurgical autografts of adrenal medulla to the right caudate nucleus in two patients with intractable Parkinson's disease; *N. Engl. J. Med.* **316** 831-834

Morihisa J M, Nakamura R K, Freed W J, Mishkin M, Wyatt R J 1984 Adrenal medulla grafts survive and exhibit catecholamine fluorescence in the primate brain; *Exp. Neurol.* **84** 643-653

Olson L 1970 Fluorescence histochemical evidence for axonal growth and secretion from transplanted adrenal medullary tissue; *Histochemie* **22** 1-7

——, Seiger A, Freedman R 1980 Chromaffin cells can innervate brain tissue: evidence from intraocular double grafts *Exp. Neurol.* **70** 414-426

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 59-68 (1990)

Morphological and Electrophysiological Criteria of the Degree of Integration of the Homo- and Heterotopic Grafts with the Host Brain

A G BRAGIN
Institute of Biophysics of the USSR Academy of Sciences Puschino 14229 (USSR)

The neocortical (N) and hippocampal (H) tissues of embryonal (E17) rats were taken in equal volumes and were grafted into barrel field (SI) of the adult rats. The morphometric, histological and electrophysiological investigations were performed 4-6 months after the grafting

Histological studies of N and H did not show significant differences in percentages of survival, the final volume and neuropil contacts between the graft and the host brain. Electrophysiological experiments revealed that the functional integration of heterotopic hippiocampal grafts was worse than that of the homotopic neocorotical grafts. This suggests the existence of some tissue-specific factors which are necessary for development of the functional connections between the graft and the host brain.

Key Words: **Morphological criterion, Histological criterion, Electrophysiological criterion, Neocortical tissue, Hippocampal tissue, Homotopic grafts, Heterotopic grafts**

Introduction

It is well known, that the embryonal nervous tissue successfully survives grafting into the brain of the newborn or adult animals, and interconnections develop between the host brain and the graft (Chang et al. 1984, Gage & Björklund 1986, Gash et al. 1985, Vinogradova 1984). There is a lot of data indicating to the possibility of recovery of impaired brain functions following transplantation of dopaminergic, cholinergic and noradrenergic tissues (Björklund et al. 1980, Gash et al. 1985, Perlow et al. 1979). Such recovery is possible because the transplanted nervous tissue is the source of neuromodulating or neurotransmitter substances necessary for normal functioning. The possibility of using neural transplantation method for compensation of function in the specific sensory areas is still unknown.

The aim of this paper is to compare morphological and neurophysiological features of the embryonal neocortical and hippocampal grafts developing in the barrel field of the adult rat neocortex.

Meterial and Methods

Transplantation

Embryos of Wistar rats taken on the 17 - 18th days of gestation (E17-18) served as the donors. The embryos were decapitated, the brain was exposed and rinsed in Eagle solution. Pieces of neocortical and hippocampal tissues were taken in equal volumes (about 1 mm^3). Recipients were the adult rats (weighing 150-180 g) of the same strain. Under Pentobarbital anaesthesia (30-40mg/kg, i.p.) the right side of the skull was exposed, and a square piece of bone (6 × 5 mm) was excised over the somatosensory area. The dura was incised and the gray matter of the barrel field was sucked out through the opening. The depth of the cavity was about 1mm; its area was about 2×4 mm^2. Coordinates of the center of the cavity were: AP= +2.0; L= 5.5. The grafts were transfered from the cold Eagle solution into the cavity with a glass capillary mounted on a syringe. The graft was placed under the incised dura, the bone was replaced over the opening in the skull, and the skin was sutured.

Recording of neuronal activity: Electrophysiological experiments were started 2 - 6 months after the grafting. Control experiments were performed in parallel on intact rats of the same age. The scalp was cut under Pentobarbital anaesthesia and metal holders for fixation of the rat head were fixed on the skull. The bone over the area of grafting was cut away. The dura was washed by warm saline and through it the presence and position of the graft was visually verified. The surface of the brain was covered by agar-agar and after its hardening by warm vaselin oil, bipolar Nichrome electrodes were inserted stereotaxically into recipient's ventrobasal complex of the thalamus (VB) for electrical stimulation. Activity of single units was recorded extracellularly by tungsten microelectrodes (tip resistance 5-10 MΩ in saline). For recording of multiple neuronal activity the microelectrodes of lower resistance (0.5-1.0 MΩ) were used. One of the skull holders was used as the reference electrode. After conventional amplification the neuronal activity was recorded on magnetic tape for photographing and computer processing later on.

Stimulation: The testing of sensory responses of the neurons was performed under light Pentobarbital anaesthesia (5 - 10 mg per kg, i.p.) in semichronic conditions. Usually two to three experiments were performed in each animal on successive days.

Contralateral vibrissae were cut to the length of 8-10 mm. Nomenclature by Woolsey and Van der Loos was used for indexing the vibrissae. For stimulation, a vibrissa with the cut-off tip was inserted into the injection, needle mounted on a relay switched by electrostimulator. Usually the duration of a vibrissa displacement was 1sec, with intervals 3-5sec, and amplitude of displacement was 3 to 5 mm. Tactile stimulation of head and body surface, as well as sound clicks, were also used.

Data processing: Neuronal activity was processed with the help of computer Electornic-60. Histograms of the interspike intervals of the first order were used to evaluate background activity; mean frequencies of discharge and variation coefficients were calculated. Peristimulus time histograms and raster displays of the series of responses were used for the analysis of effects of stimulation.

Histology: At the end of electrophysiological experiments the animal was sacrificed by an overdose of Pentobarbital and the brain was fixed and embedded in paraffine for the Nissle stain or Golgi-Cox impregnation. Careful calculations and checking of position of recorded units within the grafts were done in histological preparations with corrections for tissue shrinkage.

Results

Neocortical Grafts

The grafted neocortical tissue survived in 95% of the animals. Electrophysiological investigation was carried out in 12 grafts. EEG of nine grafts had some features typical of the EEG of the host brain. The spindle activity appearing spontaneously or evoked by tactile stimuli was observed in such grafts. Figure 1 shows an example of the graft and the host VB electroencephalogram with spindles, which follow the vibrissae deflections. The neurons of such grafts discharged spontaneous irregular spikes from 2 to 15 per sec and responded to electrical stimulation of the host VB and to tactile stimuli. Electrical stimulation of the host VB evoked responses in 59 neurons (out of 103 studied; 57%). The latencies varied from 2 to 20 ms (mean 6.0 ms). In the intact barrel field 90% of neurons responded to electrical stimulations of the VB. The range of the response latencies was 1.5 - 15 ms (means 5.6 ms). There were no statistically significant differences of latencies between intact barrel field and the grafted neurons. An example of response to electrical stimulation is presented in figure 2. The response consists of on-component, which is followed by spindle.

Deflection of the contralateral vibrissae evoked responses in 64% of the investigated cells. About half of them responded by on-off, effects. In 30% of neurons initial on-effect was followed by phasic increase of

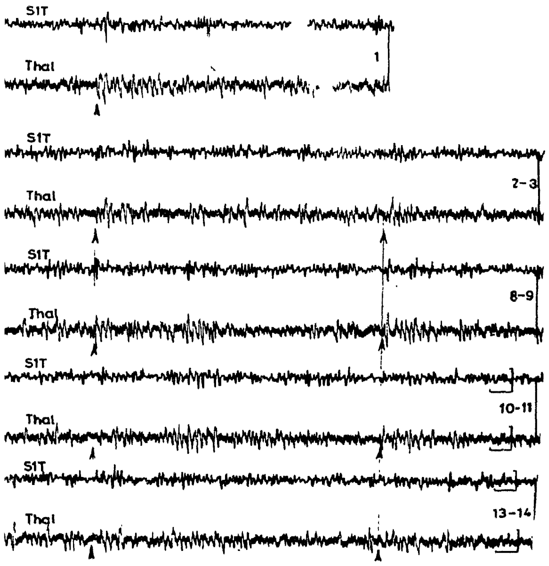


Figure 1 Electoencephalographic records in the neocortical graft (S1T) and thalamic ventro-basal nucleus (Thal) of the host brain. The arrows indicate the vibrissae deflections. Calibrations are 100 μV and 0.5 s. Figures at the right indicate the number of the stimulus application

discharge frequency, as the second component (figure 3). In some cases the second component was represented by spindle. The same types of responses were observed in the barrel field of the intact rats. We have not found any significant difference in distribution of response latencies between intact and grafted neurons.

Investigation of the properties of the receptive fields of the grafted neurons revealed that they discharged to deflection of 10 - 15 vibrissae. We have not found any neurons responding only to a single vibrissa deflection, although 10 - 30% of such neurons were present in normal barrel field. Nevertheless, we could very often find a vibrissa, which evoked response of a grafted neuron with the shorter latency than the others. That is, we could determine "the center of receptive field"

On testing the influence of tactile stimuli to other body parts of intact barrel field neurons we found about 5 to 10% of neurons responding to them (figure 4). Within the grafts the neurons responding to tactile stimulation of the body were more numerous: 38% of neurons responded to deflection of the small rostral vibrissae, 34% responded to touching the nose, 26% to touching the forepaw and 10% to touching the hindpaw (figure 4B).

The EEG of three grafts was different from the EEG of the host brain. Slow waves and spike-and-wave complexes were present in them. Spontaneous discharges of neurons in these grafts consisted of hypersynchronous bursts, and the neurons did not respond either to the electrical stimulation of the host VB or to sensory stimuli.

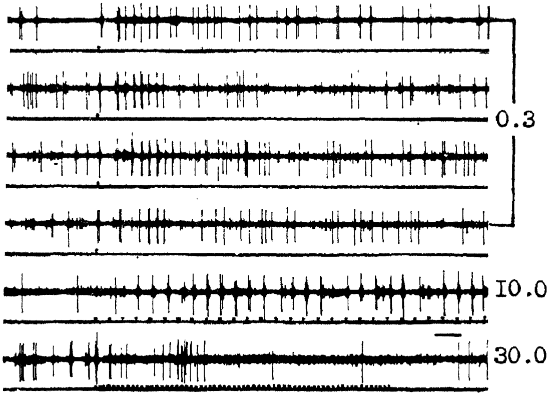


Figure 2 Response of a grafted neocortical neuron to electrical stimulation of the host VB. Figures indicate the frequency of stimulation. (Time calibration: 200 ms)

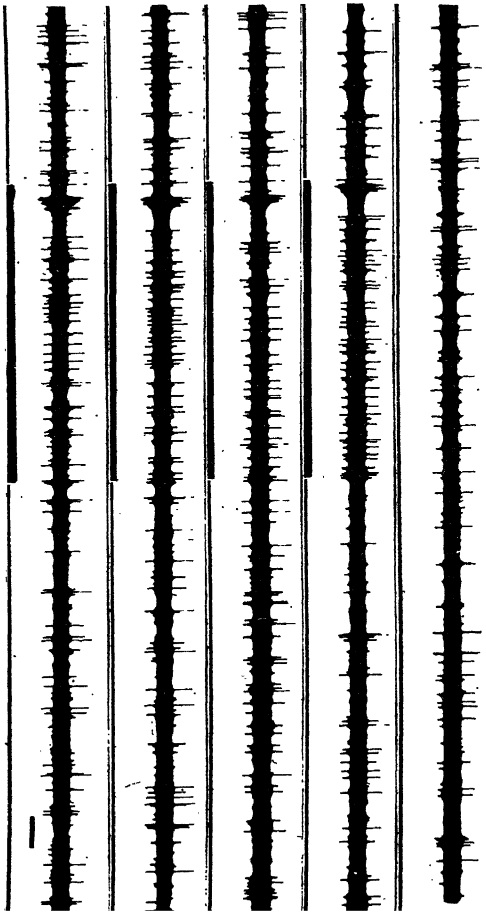


Figure 3 Background activity of the cells within the neocortical graft (upper row) and their responses to vibrissae deflection. The vibrissae deflection is indicated by thick lines under the neuronograms. (Time calibration: 200 ms)

The histological investigations did not reveal any difference between responding and non-responding grafts in volume, in number of surviving neurons and in types of neurons. But non-responding grafts were completely separated from the host brain by scar tissue. The neuropil of the responding grafts was confluent with the host brain tissue to a varying degree. The examples of two groups of grafts are presented in figure 5.

Hippocampal Grafts

The survival of the hippocampal grafts was slightly lower (85%). Their final volume was also a little lower, but differences were not significant. The background activity of the neurons consisted of single irregular spikes and of complex spikes, as in the intact hippocampus. The neurons discharging synchronously and asynchronously were revealed by registration of multiunit activity. The neurons of only four grafts (out of 10) responded to electrical stimulation of the recipient VB. The examples of the evoked neuronal population discharges are presented in figure 7. But the latencies of responses were 5 to 10 times longer, than those of the neocortical grafts. We have never observed responses of hippocampal graft neurons to tactile, or other kind of sensory stimulation of the recipient.

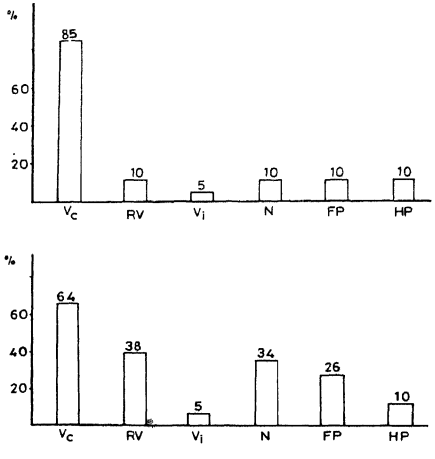


Figure 4 Diagrams of distribution of responses (in percent) to various tactile stimuli: contralateral vibrissae (Vc); rostral vibrissae (RV); ipsilateral vibrissae (Vi); nose (N); contralateral forepaw (FP); contralateral hindpaw (HP) in the intact barrel field (upper row) and in the neocortical grafts (lower row).

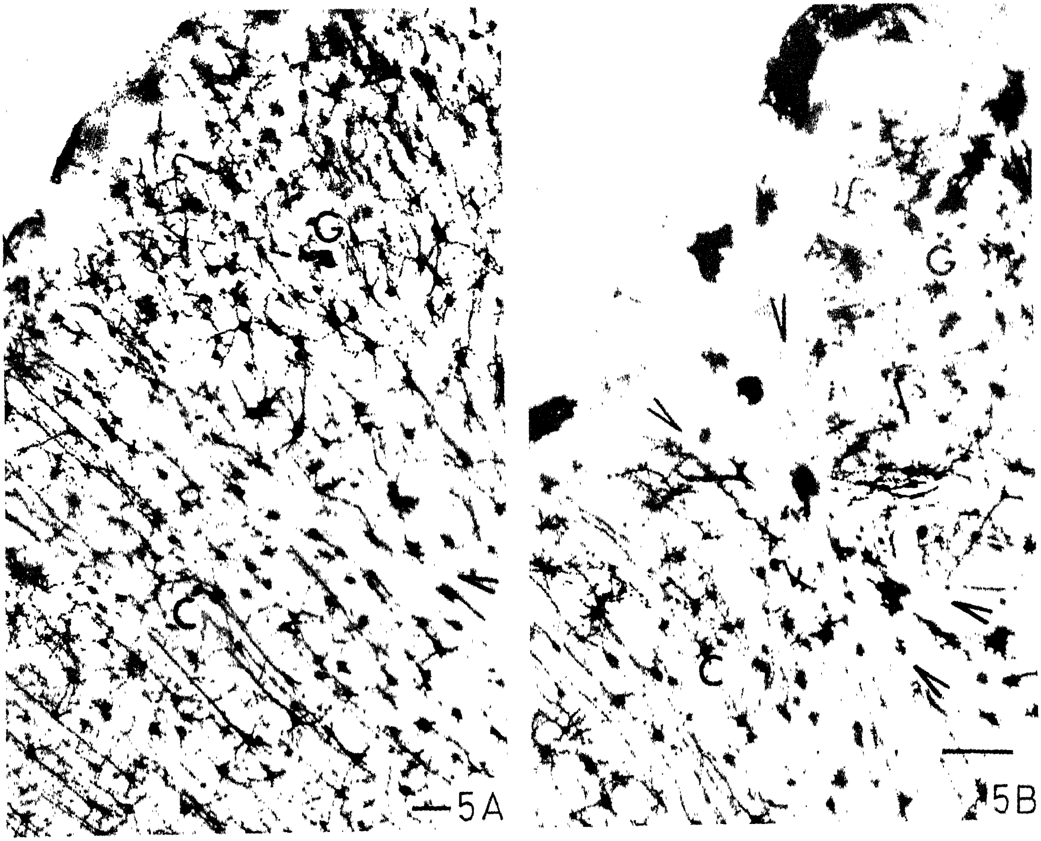


Figure 5 A & B Golgi-Cox impregnated histological sections of the host brain with the neocortical grafts. **A**, graft responding to the electrical and sensory stimuli of the host; **B**, non-responding graft. Arrowheads indicate the border between the graft and the host brain (**C**, the host brain neocortex; **G**, grafts) (Bar= 200 μm.)

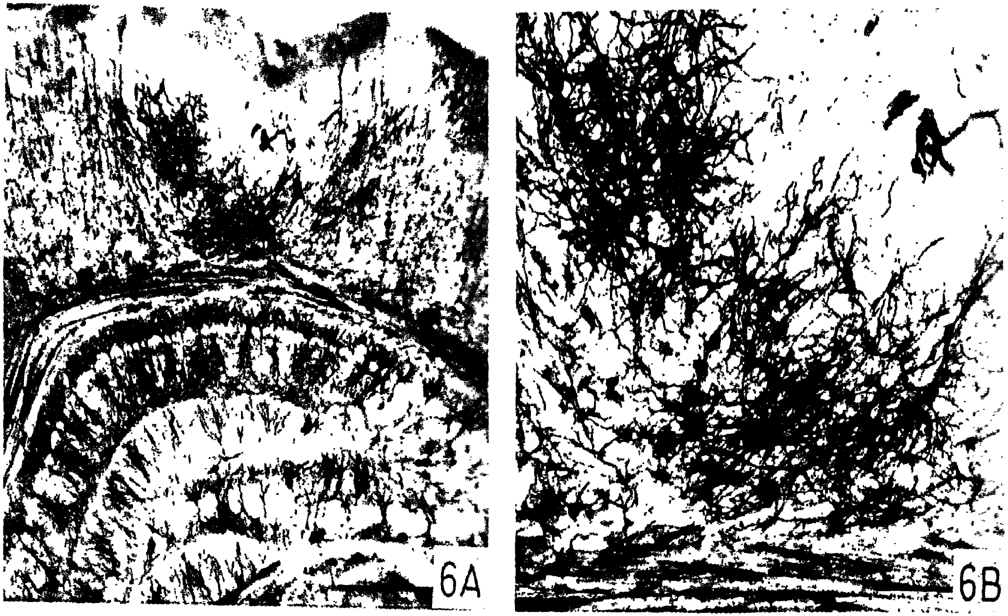


Figure 6 A & B Golgi-Cox impregnated histological sections of neocortex with the hippocampal grafts. **B**, is the larger magnification of a part of the section **A** (Bars: 1 mm in **A** and 200 μm in **B**)

The histological analysis showed, scar between non-responding hippocampal grafts and the host brain. The neuropil of the grafts, responding to electrical stimulation, was partly, or completely confluent with the host neuropil. The degree of confluence did not differ from that of the neocortical grafts. Such hippocampal graft is shown in figure 6.

Discussion

Our results confirmed that embryonal neocortex and hippocampal grafts can successfully survive within neocortex of the adult rats (Bragin 1986, Bragin et al. 1988, Girman & Golovina 1988, Levin et al. 1987, Woodruff and Baisden 1988). There was no significant difference in survival between the homo- and heterotopic grafts. The percentage of anatomically isolated grafts was somewhat greater for the heterotopic ones. However, according to the histological criteria at least half of the heterotopic grafts had the same degree of integration with the host brain, as the homotopic grafts. These morphological data might suggest that functional characteristics of the homo- and heterotopic grafts, judged on the basis of responses to electrical and tactile stimuli, may also be similar. Nevertheless, the elctrophysiological experiments revealed marked differences between the functional properties of the homo- and heterotopic grafts.

Both neocortical and hippocampal grafts retain the phenotypic characteristics of the background activity.

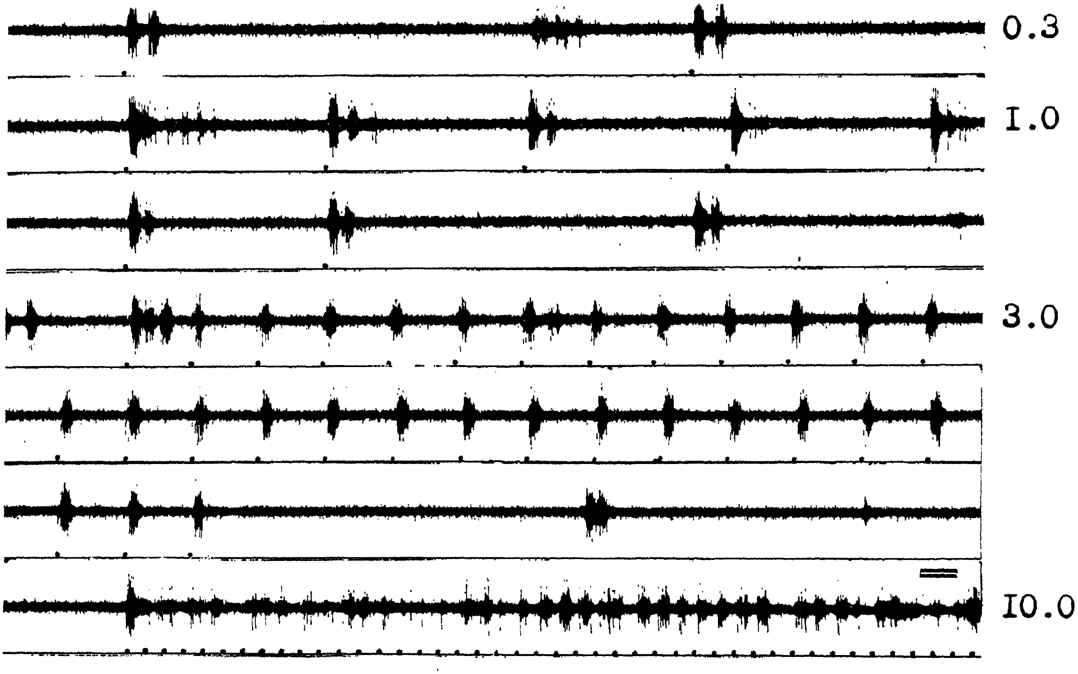


Figure 7 Responses of the hippocampal grafts neuronal population to the eletrical stimulation of the host VB. Figures at the right indicate the frequencies of stimulation (Time calibration: 200 ms).

An example of this is the presence of the complex spikes recorded from the grafted hippocampal neurons. The pathological hypersynchronous discharges were observed in some grafts. Such grafts were separated from host brain by scar, which prevents exchange of the fibres as was revealed by histological studies. It can be suggested, that within such grafts, as a result of absence of axonal exchange with the host brain, the hyperdevelopment of the intergraft synaptic connections occurs, as it happens in intraocular grafts (Zhuravleva et al. 1986). Epileptic discharges resulted from such isolated development (Bragin & Vinogradova 1983, Bragin et al. 1985).

The hypersynchronous discharges were absent in 75% of the neocortical and in 50% of the hippocampal grafts, which had no scar at the broder with the host brain. The neocortical grafts established functional connection with the host brain. Their neurons discharged both to electrical stimulation of the host VB and to tactile stimuli. There were no significant differences in latencies of the responses between grafted and normal neurons within the barrel field. It should be emphasized that the neurons with the monovibrissal receptive fields were absent within the grafts. Much wider convergence of the body surface upon the grafted neocortical neurons in comparison to the intact barrel field neurons was observed. From this point of view the grafts resembled the 2nd somatosensory area or SI of the young animals (Armstrong-James 1975, Carvell & Simons 1986).

Although 50% of the hippocampal grafts had no scar at the border with the host brain, they were much less reactive to the electrical stimulation of the host VB than the neocortical grafts. The latencies of responses of the hippocampal neurons to VB stimuli were longer, and they did not discharge at all to tactile stimulation of he host.

Thus, though according to the histological data some of the heterotopic grafts do not differ from homotopic ones in the degree of integration, the established functional connections are much more limited as shown by the electrophysiological testing. The causes of these differences are not clear yet. It is possible that limited functional integration of the heterotopic grafts depends upon inadequate tissue-specific factors preventing development of effective synaptic contacts with the host brain. As to the homotopic grafts, they established effective functional connections with the recipients's brain, and it can be assumed that they can participate in the processing of sensory information, received by the host.

References

Armstrong James M 1975 The functional status and columnar organization of single cells responding to cautaneous stimulation in neonatal rat somatosensory cortex S1; *J. Physiol. Lond.* **246** 501-538

Björklund A, Dunnett S B, Stenevi U, Lewis M E and Iverson S O 1980 Reinnervation of the denervated striatum by substantia nigria transplants: functional consequences as revealed by pharmacological and sensorimotor testing; *Brain Res.* **199** 307-338

Bragin A G 1986 Participation of the neurons of the embryonal rat neocortax grafts in sensory functions of the recipient neocortex; *Zhurn. Vyss. Nervn. Deyat.* **36** 930-938 (in Russian)

——, Vinogradova O S 1983 Comparison of neuronal activity in septal and hippocampal grafts developing in the anterior eye chamber of the rat; *Devel. Brain Res.* **102** 273-286

——, ——, Mironov S Ph 1985 Neuronal activity of septal and hippocampal grafts developing separately and together in the anterior eye chamber of the rat; *Neurophysiologia* **17** 61-69 (in Russian)

——, Bohne A, Vinogradova O S 1988 Transplants of the embryonal rat somatosensory neocortex in the barrel field of the adult rat: responses of the grafted neurons to sensory stimulation; *Neuroscience* **25** 751-758

Carvell G E and Simons D J 1986 Somatotopic organization of the second somatosensory area (SII) in the cerebal cortex of the mouse; *Somatosensory Res.* **3** 213-237

Chang F F, Steedman J G, Lund R D 1984 Embryonic cerebal cortex placed in the occipital region of newbom rat makes connections with the host brain; *Dev. Brain Res.* **13** 164

Gage F H and Björklund A 1986 Neural grafting in the aged rat brain; *Ann. Rev. Physiol.* **48** 447-459

Gash D M, Collier T J and Sladek J R 1985 Neural transplantation: A review of recent developments and potential application to the aged brain; *Neurobiol. Ageing* **6** 131-150

Girman S V and Golovina I·L 1988 Electrophysiological study of the afferent connections of the embryonal neocortex grafted into projection areas of neocortex of the adult rats; *Neurophysiologia* **20** 448-456 (in Russian)

Jaeger C B and Lund R D 1979 Efferent fibers from transplantated cerebral cortex of rats; *Brain Res.* **165** 338-342

Levin B E, Dunn-Meynell A and Sved A F 1987 Functional integration of fetal cortical grafts into the afferent pathway of the rat somatosensory cortex (SmI); *Brain Res.* **19** 723-734

Perlow M J, Freed W J, Hoffer B J, Seiger A, Olson L, Wyatt R J 1979 Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system; *Science* **204** 643-647

Vinogradova O S 1984 Development of the mammalian nervous tissue transplanted into the brain and anterior eye chamber: Problems and perspectives; *Ontogenesis*, **15** 229-251 (in Russian)

Woodruff M L, Baisden R H 1988 Structure and function of initracerebral grafts of fetal neocortical or hippocampal tissue; in: Neural Plasticity: *A Lifespan Approach. Alan R. Liss. Inc.* 69-104

Zhuravleva Z N, Bragin A G and Vinogradova O S 1986 Organization of the nervous tissue (hippocampus and septum) developing in the anterior eye chamber. III. Axonal processes and their synaptic endings; *J. Hirnforschung* **27** 323-341

Proc. Indian natn. Sci. Acad **B56** No. 1 pp. 69-72 (1990)

Alterations in Neuronal Development under Influence of Conditions of Nurture and Heterotopic Transplantation

T DESIRAJU
Department of Neurophysiology, National Institute of Mental Health and Neuro-sciences, Bangalore 560029 (India)

(1) Impoverished nurture created through undernutritional condition caused significant deviations in the dendritic arborization and numerical spine densities of pyramidal neurons of visual and motor cortices in developing rat brain. The development of spine numbers lagged behind in the initial postnatal ages, but later on the numbers increased and continued at a level higher than the control without having been pruned while attaining the adult age. This was interpreted as a homeostatic adaptation response of the neurons in the brain to the demands of undernourished state. (2) The enriched experience provided through operant learning sessions in the normally nourished developing rat pus also caused certain increments in the spine densities of specific regions of dendrites of hippocampal CA3 neurons, i.e., implying that only some inputs to the neurons might have been involved in providing the stimulus for the spine genesis in the developing brain. (3) The hippocampus transplant that matured under heterotopic environmental conditions showed significantly reduced spine densities as well as dendritic branching, in yet another manner. However, several of these grafted pyramidal neurons have more exuberant branching of apical dendrites and high spine densities, even better than the native control, while the basal dendrites showed the opposite trend of development. Hence the alterations in the transplants may not be simply due to the effect of a new environment, but may be due to complex interactions of both growth promoting and pruning factors within the neurons and without. (4) Thus, the three models of experimentation revealed the scope and variety of alterations of the neuronal phenotypes being the products of expression of the genetic and epigenetic or environmental influences.

Key Words Developmental neuronal plasticity, Nurture moulding neurons, Neural transplants, Learning and neurons, Brain growth under undernutrition

Introduction

In recent years, knowledge on the factors that alter the development and maturation of neuronal connections and synapses has been rapidly advancing. It has been revealed that there is a significant degree of plasticity in the shaping of neuronal connectivity. The studies presented in this paper contribute information from experimental model approaches to assess the modifiability of dendritic branching and spine genesis of cerebral cortical neurons (both neocortex and limbic cortex), by varying either the material conditions of nurture (diet restriction) or by varying experiential opportunity made available to the subject during the age of brain growth and development. Further more, this report also presents information from another model of study on the type of changes that might occur in neurons when neural tissuses are transplanted from their native region into new sites of nurture. These studies are intended to advance the understanding of the conditions that induce the expression of plasticity of neurons as well as the nature. of the response.

Materials and Methods

All the experiments have been done on Wistar rats. The details of the methods of nutritional deprivation and assessment of resulting alterations of dendritic arborizations and spine numbers of neocortical pyramidal neurons have been described earlier (Gundappa & Desiraju 1988, Mascarenhas et al. 1986, Rajanna et al. 1987). The method of providing new or enriched experience through operant conditioning and assessing consequential changes of dendritic branching and spine densities of hippocampal pyramidal neurons was described in detail earlier (Mahajan & Desiraju 1988). The methodological detail of transplantation of embryonic hippocampal neural tissue into neocortex of juvenile recipient, and the assessment of the changes of dendrites and spines of neurons in the grafted tissue was also published earlier (Murthy & Desiraju 1989). The method of electronmicroscopic analysis of alterations of synapses in EPTA treated cortical tissue has been also described elsewhere (Murthy et al. 1989).

Results

Deprived (nutrition) condition: The ontogenetic processes of exuberance and pruning of dendritic spines of pyramidal neurons of neocortex (lower laminae of visual and motor areas) were firstly characterised in rapid Golgi studies in developing Wistar rats reared under normal conditions. Secondly, the effect of changing the condition of nurture by depriving nutrition (caloric) during gestational and post-natal age was also studied (Gundappa & Desiraju 1988).

In the ontogeny of normally nourished group, exuberant spine densities (numbers) reached between 26-50 days of age and underwent a progressive decline or pruning upto about 50% by adult age, having certain differences among different categories of dendrites (basal, apical), and between visual and motor cortical neurons. The patterns of ontogenetic changes of the neurons of undernourished group were also similarly assessed and compared. The undernourished neuronal ontogeny was significantly altered, in that there was an initial lag in the spine densities, followed by an increase in the spine densities which continued upto the adult age, instead of the normally expected pruning. This deviation in the pattern due to deprivation in nurture during the early age could not be prevented by late postnatal restitution of normal nutrition. Number of the EPTA stained synapses in the molecular layer of the undernourished rat brain cortices also showed similar patterns of deviation (Murthy et al. 1989).

Learning experience: In another experimental approach, enriched experience was provided to 18-day old rat pups in operant conditioning paradigm for food reward for a few days, and the changes of dendritic branching, and spine densities of hippocampal pyramidal neurons of CA3 region were assessed. The data revealed significant increases in the dendritic branching of the hippocampal neurons and spine densities of some specific segments, thereby showing that the enriched experience altered neuronal development (Mahajan & Desiraju 1988).

Transplant Development: In another approach of study, embryonic (18-day) hippocampal tissue was transplanted into neocortical cavity in 18-day-old rat pup to study the extent of differentiation and development of neurons in a heterotopic environment. Such a study was expected to contribute to the understanding of the scope of plasticity in neuronal development (Desiraju 1989). The results showed that the hippocampal neurons (granule and pyramidal cells) developed apparently normal forms, but with significantly reduced dendritic branching and spine densities in some neurons compared to native hippocampal neurons (Murthy & Desiraju 1989). There were also aberrations in the forms of neurons, The basal dendrites of the CA3 neurons had significantly less branching and spines than control, while the opposite trend of exuberence was observed with apical dendrites.

Discussion

The above studies revealed that the differentiation and development of cortical neurons can be significantly altered in different ways by different conditions of nurture. They have a wide range of plasticity. In the subjects nurtured on undernutrition, the computerized electrographic power spectral analysis revealed major deviations from normal (Rajanna et al. 1987). The levels of dopamine, noradrenaline and serotonin of different brain regions of these subjects were also significantly altered (Annamma and Desiraju 1988). Hence, it was obvious that the undernutritional nurture caused significant alterations in not merely the cortical pyramidal neuronal dendrites, but in the larger networks of the developing brain. These are probably homeostatic adaptation responses. Alterations in the neurotransmitter content of different brain regims (dopamine, noradrenaline, serotonin, glutamate and GABA) were also observed in the brains of rats consuming lead during postnatal brain development (Shailesh Kumar & Desiraju 1990).

The increase in number of spines in specific regions of dendrites in learning experiments indicated that transmission was augmented for some afferent inputs due to tenriched early learning. The transplanted neurons having been disrupted of afferent connections responded in a different manner.

From the observations it is clear that the various conditions of nurture, both of material kind and of subjective experimential kind will critically determine the shaping of the brain neural networks and their functioning perhaps within the limits of genotypic possibilities of individuals. In the foreseable future, further informations in this area will lead to development of special applications that can enable to mould the growth and expression of the potentials in each individual, and also to prevent maladaptations or to devise appropriate therapeutic methods to correct some of the ills.

References

Annamma C and Desiraju T 1988 Deviations in Brain Development of F2 generational calorie undernutrition and scope of their prevention by rehabilitation: Imbalances in the levels of noradrenaline, dopamine and serotonin in different brain regions; *Biogenic Amines* **5** 323-337

Desiraju T 1989 Grafting of neural tissue to remedy neural damage; in *Molecular Mechanisms underlying Neuronal Response to Damage* eds B S S R Rao and S C Bondy (Bangalore: NIMHANS)

Gundappa G and Desiraju T 1988 Deviations in brain development of F2 generation on caloric undernutrition and scope of their prevention by rehabilitation: Alterations in dendritic spine production and pruning of pyramidal neurons of lower laminae of motor cortex and visual cortex; *Brain Res* **456** 205-223

Mahajan D S and Desiraju T 1988 Alterations in dendritic branching and spine densities of hippocampal CA3 pyramidal neurons induced by operant conditioning in the phase of brain growth spurt; *Exp Neurol* **100** 1-15

Mascarenhas C, Rajanna B, Gundappa G, Cherian A and Desiraju T 1986 Experimental findings on the impact of early undernutrition on brain development and effects of subsequent rehabilitation; in *Iodine Nutrition, Thyroxine and Brain Development* pp 181-199 eds N Kochhu Pillai M Karmarkar and V Ramalingaswami (New Delhi: Tata McGraw-Hill)

Murthy K D, Gundappa G, Sulekha S and Desiraju T 1989 Alterations of synapse numbers in undernourished developing cerebral cortical regions; in *Molecular Mechanisms Underlying Neuronal Response to Damage* eds B S S R Rao and S C Bondy (Bangalore: NIMHANS)

Murthy S K and Desiraju T 1989. Quantitative assessment of dendritic branching and spine densities of neurons of hippocampal embryonic tissue transplanted into juvenile neocortex; *Dev Brain Res* **46** 33-45

Rajanna B, Mascarenhas C and Desiraju T 1987. Deviations in brain development due to the caloric undernutrition and the scope of their prevention by rehabilitation: Alterations in the power spectra of EEG of areas of neocortex and limbic system; *Dev Brain Res* **37** 97-113

Shailesh Kumar M V and Desiraju T 1990 Regional alterations of brain biogenic amines and GABA/glutamate levels in rat following chronic lead exposure during neonatal development; *Arch Toxicol* **65**

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 73-84 (1990)

Intracerebral Transplantation of Cultivated Glio-Neuronal Aggregates and Fetal Brain Tissue Fragments

ILYA V VICTOROV* and ANATOLY A LYJIN
Brain Research Institute, All-Union Research Center for Mental Health, Academy of Medical Sciences of the USSR, Moscow (USSR)

Cultivated fragments of fetal brain tissue and glio-neuronal aggregates survived intracerebral transplantation (survival rate 90-100%) and were completely integrated with the host brain. Histological studies showed a high degree of neuronal differentiation and cytoarchitectural organization of the transplants. However, only some of the transplanted glio-neuronal aggregates showed neuronal organization typical of dissociated brain structure. In contrast, practically all of the transplants of cultivated brain tissue were characterized by a mature organotypic (cytoarchitectural) development. These results suggest that fetal brain tissue and glioneuronal aggregates of dissociated brain cells can be used for intracerebral transplantation after roller cultivation.

Key-Words: Intracerebral transplantation, Glio-neuronal aggregates, Fetal brain, Neuronal Differentiation

Introduction

It is now well documented that fetal brain tissue or dissociated cells transplanted into the brains of adult animals survive and integrate with the brain of the recipient and serve as a basis for functional recovery of damaged brain (Wallace & Das 1983; Sladek and Gash 1984, Bjorklund & Stenevi 1985). In most of the transplantation studies, freshly dissected fetal brain tissue or dissociated fetal brain cells were used as the source of donor material. However, recently several experiments have been carried out using fetal brain tissue or cells after a preliminary cultivation *in vitro* for varying periods of time as material for intracerebral transplantation (McLoon et al. 1981, Bunge et al. 1983, Brundin et al. 1984, Lindsay et al. 1984, Bernstein & Goldberg 1986, Gibbs et al. 1986, Colombo et al. 1987). It is necessary to emphasize that in these studies nerve cells and tissue cultures were grown on a solid substrate.

The purpose of the present study was to determine the possibility for intracerebral transplantation of glio-neuronal aggregates or organotypic fragments of fetal brain tissue after short-term cultivation in a mini-roller system (Victorov et at. 1985).

Materials and Methods

Subjects: Sixty-eight adult (6-12 month old) Wistar rats were used. The rats were housed in groups of 6 animals in the vivarium with constant temperature and humidity on a 12 : 12 hr lighting schedule and with *ad libitum* access to food and water.

**Present address:* Laboratory of Experimental Neurocytology, Brain Research Institute, Pereulok Obucha 5, Moscow 103064 (USSR)

Dissociation of fetal brain tissue: Different structures were dissected aseptically from the brains of 16-20 day old rat fetuses (hippocampus, cerebellum, neocortex, olfactory bulb) and mechanically dissociated in sterile Simms' X7 balanced salt solution (SBSS) by repeated aspiration of minced brain tissue through a fire-polished Pasteur pipette. A suspension of dissociated cells was transferred to a centrifuge tube, after removing undissociated pieces of tissue, and centrifuged for I min at 3000 rpm. The pellet was resuspended in the following culture medium: fetal calf serum 5%, human placental serum 5%, Eagle's Minimal Essential Medium 70%, SBSS 20%, glucose 600 mg%, insulin 0.2 U/ml, HEPES buffer 10 mM.

. *Preparation of brain tissue fragments*: The desired brain regions were dissected aseptically from rat fetuses. Small (I-2 mm^3) fragments of tissue were cut into sterile SBSS. After a brief wash in fresh SBSS, the brain fragments were transferred to the same culture medium as above.

Culture system: High-spead mini-roller (figure 1) mounted inside a water-jacked incubator were used to culture both fetal brain tissue and glio-neuronal aggregates (Victorov et al. 1985). Two milliliters of culture medium containing 20–30 tissue fragments or an equal volume of dissociated cell suspension were transferred into 10 ml culture bottles. The bottles were plugged with rubber stoppers and put on the mini-roller. The cultures were maintained under constant rotation (70 rpm) at 35±5 0,5°C up to 9 days without changing the culture medium.

Transplantation of cultures: Three to nine-day old glioneuronal aggregates obtained from a suspension of dissociated fetal brain cells or brain tissue fragments were used for intracerebral transplantation. Following a short wash in sterile SBSS the neural explants were aspirated into the tip of a capillary glass pipette (inner diameter –700±100 μm) connected to a microsyringe filled with SBSS. The recipient rat was anesthetized with chloral hydrate (i.p., 330 mg/kg) and placed in a

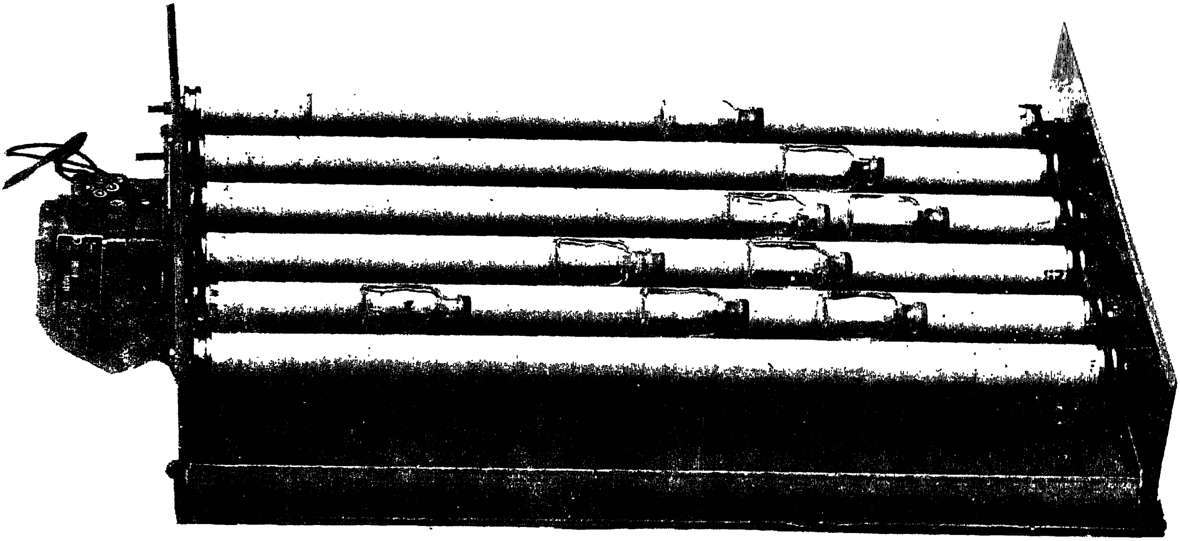


Figure 1 High-speed mini-roller with bottles for roller cultivation of glio-neuronal aggregates and fetal brain fragments.

stereotaxic frame. The glass pipette was inserted into the different regions of the hosts brain (striatum, neocortex, hippocampus, ventricles etc.) through 1. 5—2 mm burr hole in the dorsal aspect of the skull. The transplants were injected under pressure with a microsyringe and 3-5 min were allowed for the ejection of liquid and stabilization of transplants before slowly withdrawing the pipette. The stereotaxic coordinates used were of Paxinos and Watson (1982)

Morphological studies: The recipient rats were sacrificed by decapitation under deep ether anesthesia. The brains were removed and fixed either in formaline-acetic acid-alcohol mixture (2:1:7) for subsequent paraffin embedding and cresyl violet (Nissl) staining of serial sections, or in 10% neutral formaline for silver impregnation according to the modified Bielschowsky method (Victorov 1969). Kluver-Barrera and Golgi methods were also used. Indian ink transcardial perfusion was used to study the development of blood vessels and angioarchitecture of the transplants. Some frozen sections were counterstained with cresyl violet to verify the results.

Results

Transplantation of Glio-neuronal Aggregates Glio-neuronal aggregates of fetal brain cells assume a spherical form after 1-2 days of roller cultivation, characterized by small size (up to 500 μm in diameter) and loose cell structure. Undifferentiated neuronal and glial cells were randomly interspersed in the sphere. As a rule, glio-neuronal aggregates grafted after 1-2 days *in vitro* (DIV) did not survive in the host brain tissue: only local gliosis could be seen at the site of the transplant two weeks after transplantation.

The glio-neuronal aggregates increased in size (0. 7-1 mm) and consisted of closely packed neuronal and glial cells after 3-5 DIV (figure 2A). Some of the aggregates, especially those formed from dissociated cells of the neocortex, showed distinct signs of cytoarchitectural differentiation in the form of superficial stratified cortical plate-like layer of undifferentiated neurons and "subcortical white matter" glial cells. When the recipient brains were examined 1-3 months after transplantation, the majority of these glio-neuronal aggregates appeared as a group of well-differentiated nerve cells located in a small area of the host brain. The transplanted aggregates displayed variability in the size and number of neurons and in their cytoarchitectural patterns. This made it difficult to discern any laminar or nuclear cytoarchitectural formations (figure 3). As a rule, the glio-mesodermal scar was absent at the border between the transplant and the host brain except when the transplants were located in close proximity to the surface of the neocortex. The transplanted tissue appeared to be fully integrated with the host brain. It was possible to follow silver stained nerve fibers passing across the border of the transplant into the host brain (figure 4).

In the case of intraventricular localization, glio-neuronal aggregates (3-5 DIV) became attached, as a rule, to the ventricular wall. Following the disappearance of the ependymal cell layer, a zone of parenchymal confluence could be seen (figure 5). The presence of this zone is an indication of structural integration between the transplanted glio-neuronal aggregate and the host brain. This type of partially integrated intraventricular transplants showed good viability and highly differentiated cellular and cytoarchitectural organization (figure 5).

After 6-9 day in culture the glio-neuronal aggregates were characterized by more mature neurons and, in some cases, more pronounced cytoarchitectural organization. The mean size of these aggregates was 1 mm in diameter, and they tended to form multi-aggregate conglomerates. Beyond 8-9 day darkly stained piknotic cells could be seen in the core of some glio-neuronal aggregates.

The glio-neuronal aggregates transplanted after 7-9 DIV survived and were wholly integrated into the hosts brain (figure 6). These transplants achieved a high level of cytological and cytoarchitectural differentiation, though in some cases it was possible to observe signs of partial neuronal degeneration following transplantation (up to the second week) with more pronounced glial reaction in the host tissue surrounding the transplants during later stages of survival.

Intraparenchymal Transplantation of Cultured Fragments of fetal Brain: At the time of transplantation, the fragments of fetal brain consisted of neuroblasts, neurons in the early stages of differentiation and immature glial cells. The cellular composition and cytoarchitectural organization of the transplants were similar to that seen within the donor brain (for example, after 2 DIV the cerebellar fragments taken from 17-day-old

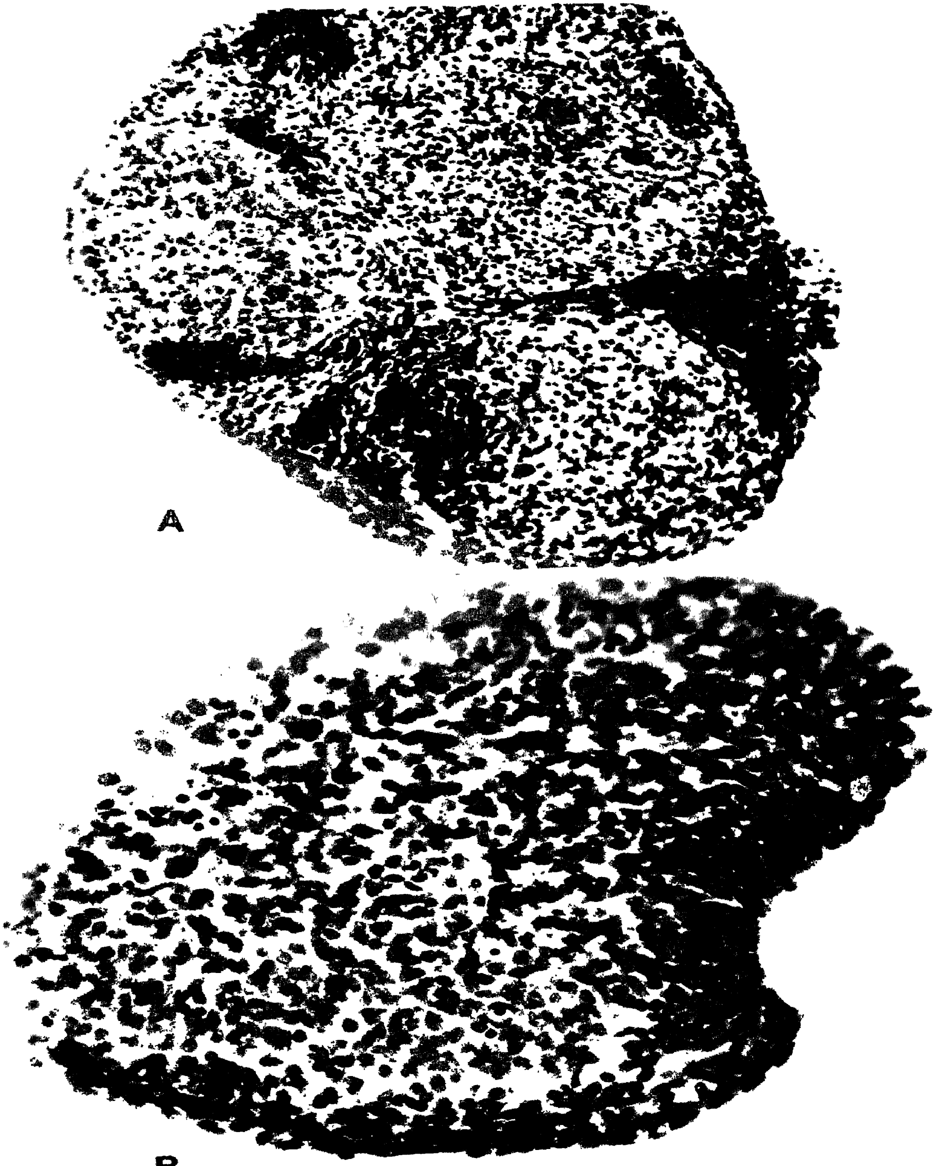


Figure 2 **A** Section through an aggregate of dissociated cerebellar cells taken from 18-day rat embryo. 4 days of roller cultivation. Cresyl violet stain. (Scale: 100 μm);, **B,** Section through a tissue fragment of cerebellum taken from 17-day rat embryo. 1 day of roller cultivation. Cresyl violet stain. (Scale: 50 μm).

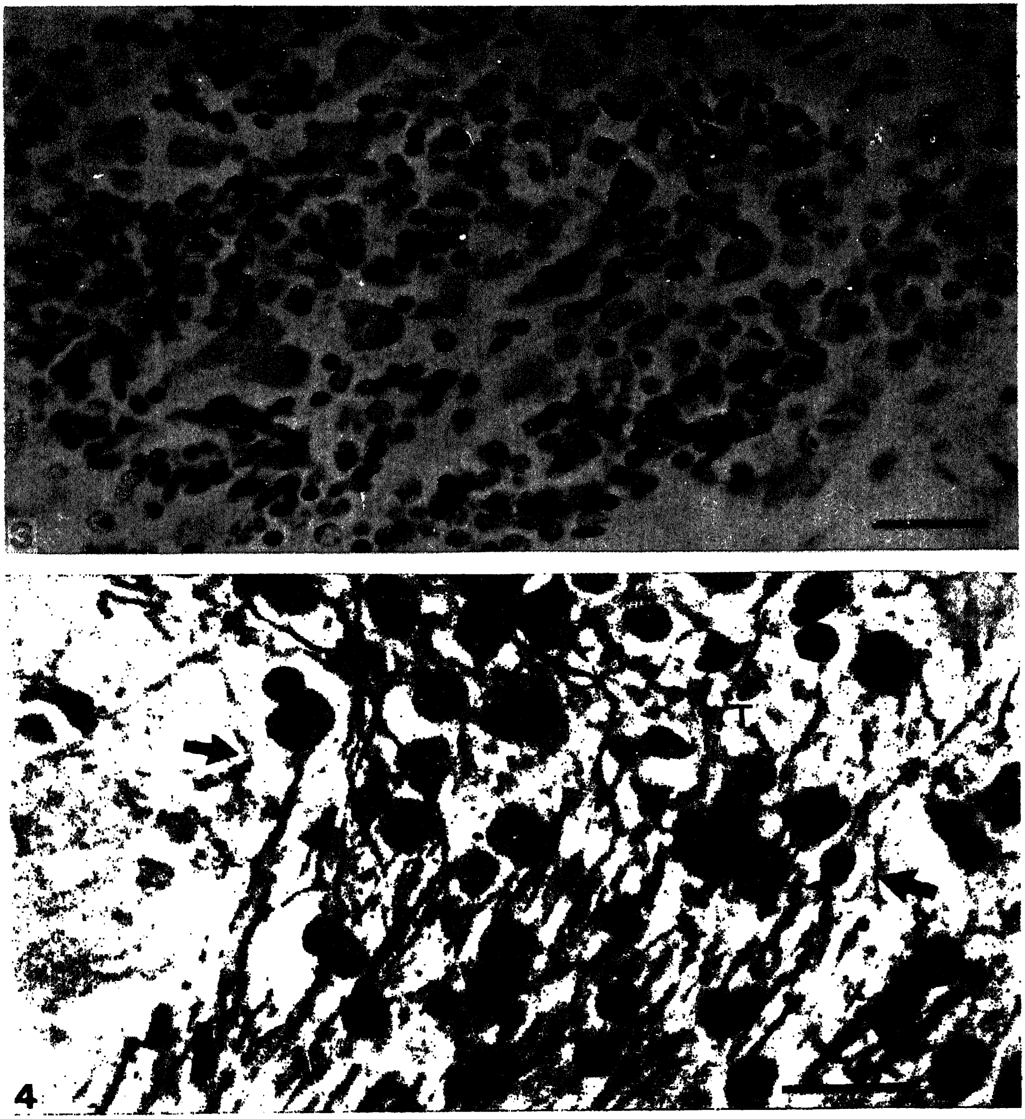
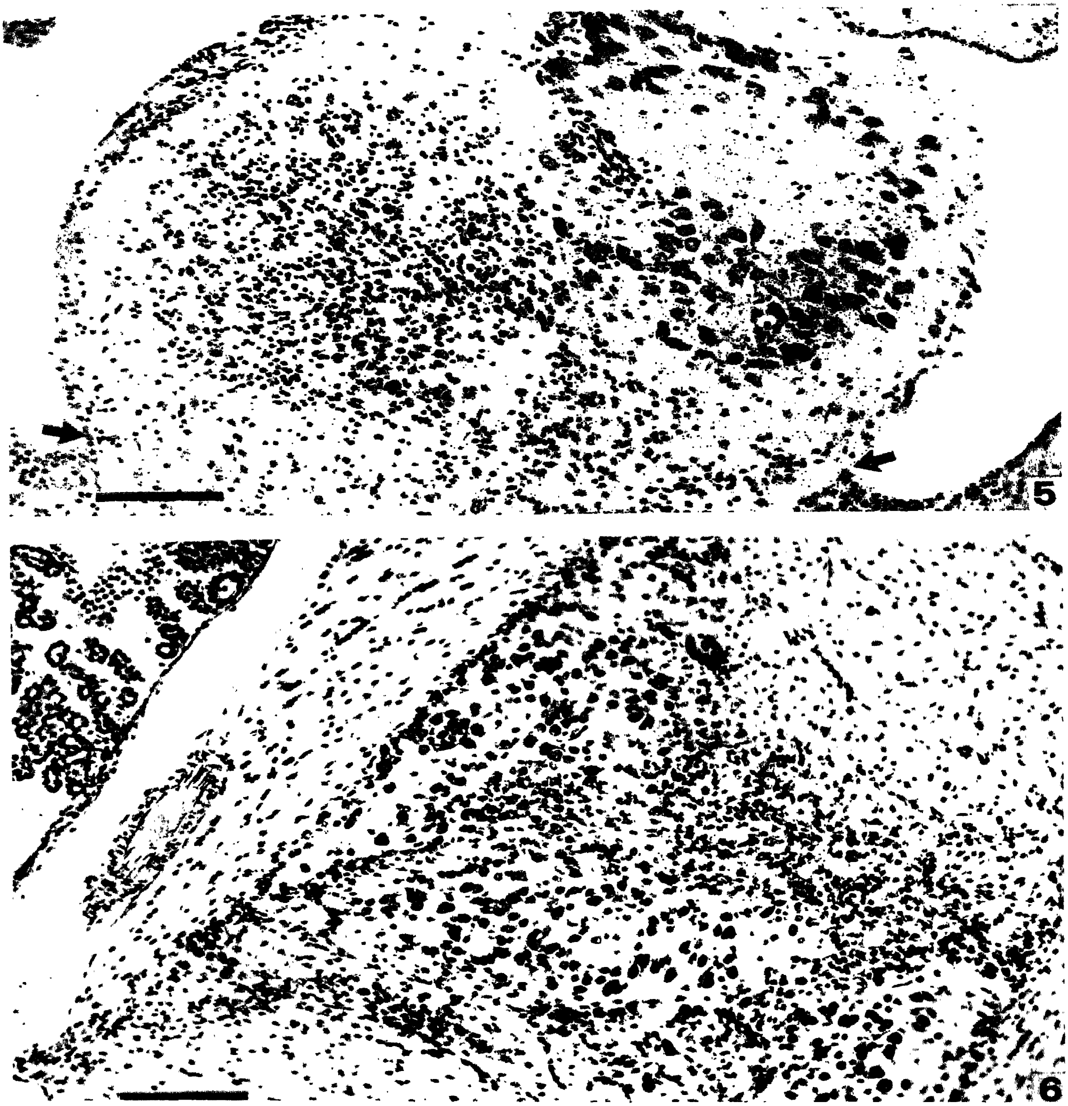


Figure.3-4 3, Glio-neuronal aggregate of cerebellar cells from 20-day rat embryo transplanted into the striatum of an adult rat after 4 days of roller cultivation. 14 days survival after transplantation. Cresyl violet stain (Scale: 25 μm) 4, Glio-neuronal aggregate of the whole forebrain dissociated cells from 17-day rat embryo transplanted into the striatum of an adult rat after 3 days of roller cultivation. 65 days survival after transplantation. Bielschowsky-Victorov silver impregnation. Note the axons passing the border of the transplant (arrows) into the recipient brain. T-transplant. (Scale: 25 pm.)



Figures 5-6 5, Glio-neuronal aggregate of dissociated cerebellar cells from 18-day rat embryo transplanted into the lateral ventricle of an adult rat brain after 4 days of roller cultivation. Note the separate localization of Purkinje and granule cells in transplanted aggregate and complete confluence of the transplant and the recipient brain (arrows). 72 days survival after transplantation. Cresyl violet stain. (Scale: 150 μm); **6**, Glio-neuronal aggregate of dissociated neocortical cells from 17-day rat embryo transplanted near the internal capsule after 9 days of roller cultivation. 14 days survival after transplantation. Cresyl violet stain. (Scale: 150 μm.)

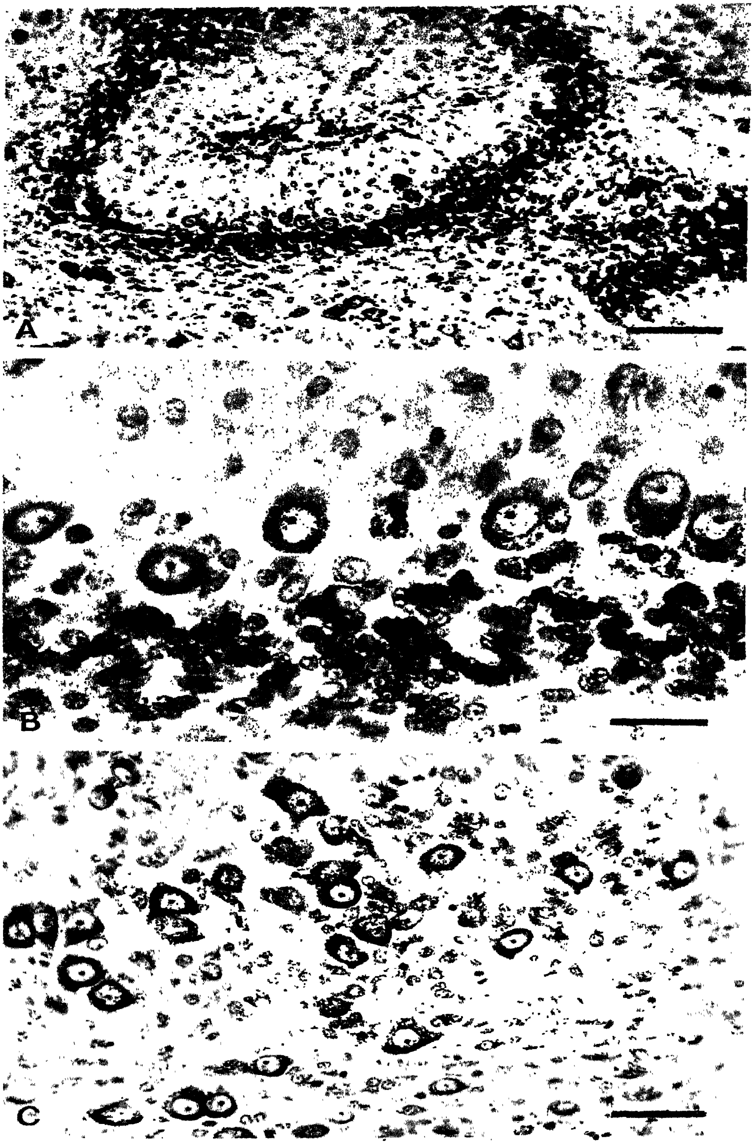


Figure 7A-C A, Tissue fragment of cerebellum from 17-day rat embryo transplanted into the cerebellar cortex of an adult rat after 1 day of roller cultivation. 26 days survival after transplantation. (Scale: 100 μm); **B.** *Cytoarchitecture of the cerebellar transplant shown in "A": organotypic localization of Purkinje and* granule cells in the cortical part of the transplant. (Scale: 25 μm.) **C**, Cytology of the subcortical region of the cerebellar transplant shown in "A". Note the normal appearance of multipolar neurons of cerebellar nuclei (A,B,C,—Cresyl violet stain).

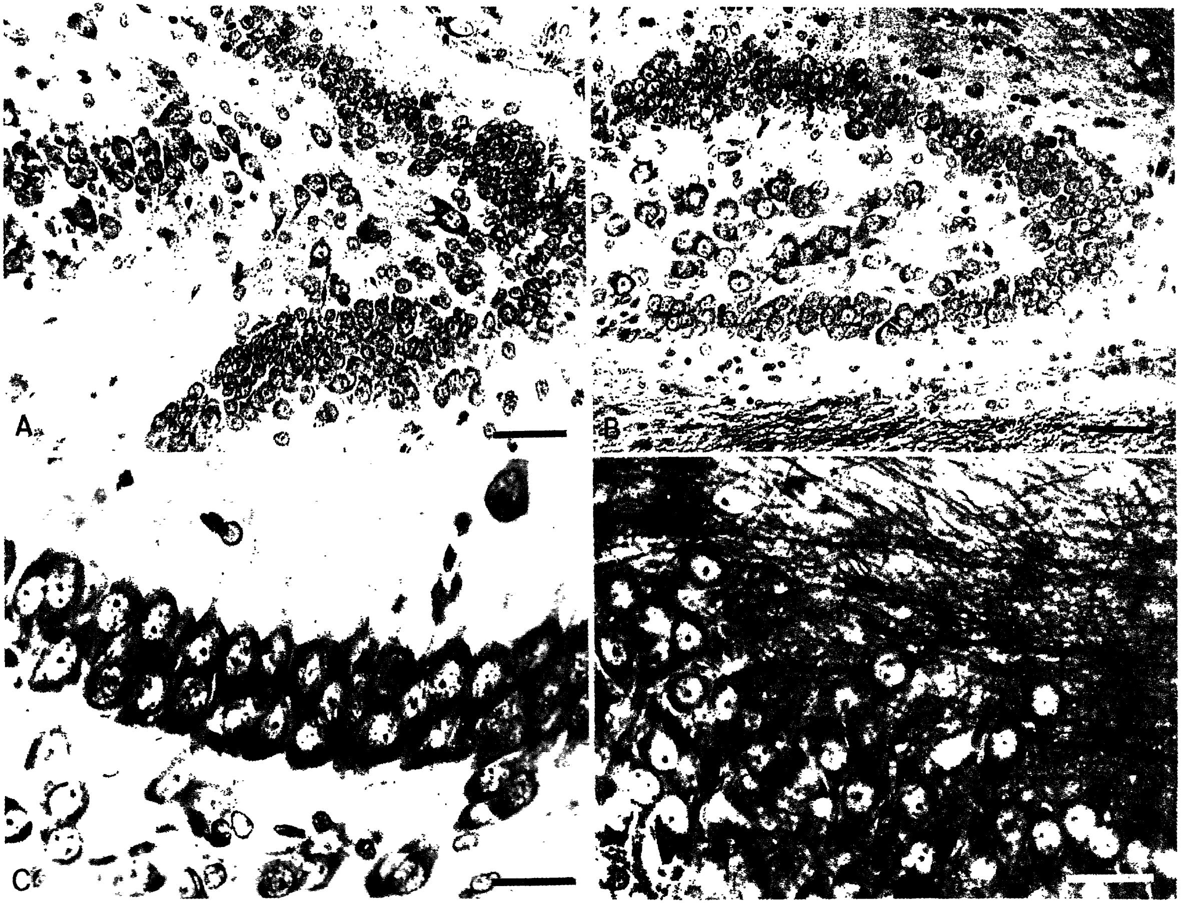


Figure 8 A-D 8 Cytoarchitecture and cytology of hippocampal tissue fragment from 17-day rat embryo transplanted into the hippocampus of an adult rat after 4 hours of roller cultivation. 46 days survival after transplantation. **A, B**, Organotypical neuronal organization of the Fascia dentata and CA_3, CA_4 fields of the cultivated hippocampal transplant. Cresyl violet stain (Scale: 50 μm). **C**, Detail of the hippocampal transplant shown in "A": pyramidal neurons of the CA_3 field. Cresyl violet stain. (Scale: 25 μm). **D**, Silver stained section of the hippocampal transplant shown in "B": pyramidal neurons of the CA_3field and dense network of nerve fibers. Bielschowsky-Victorov silver impregnation and cresyl violet couterstaining. (Scale: 25 μm).

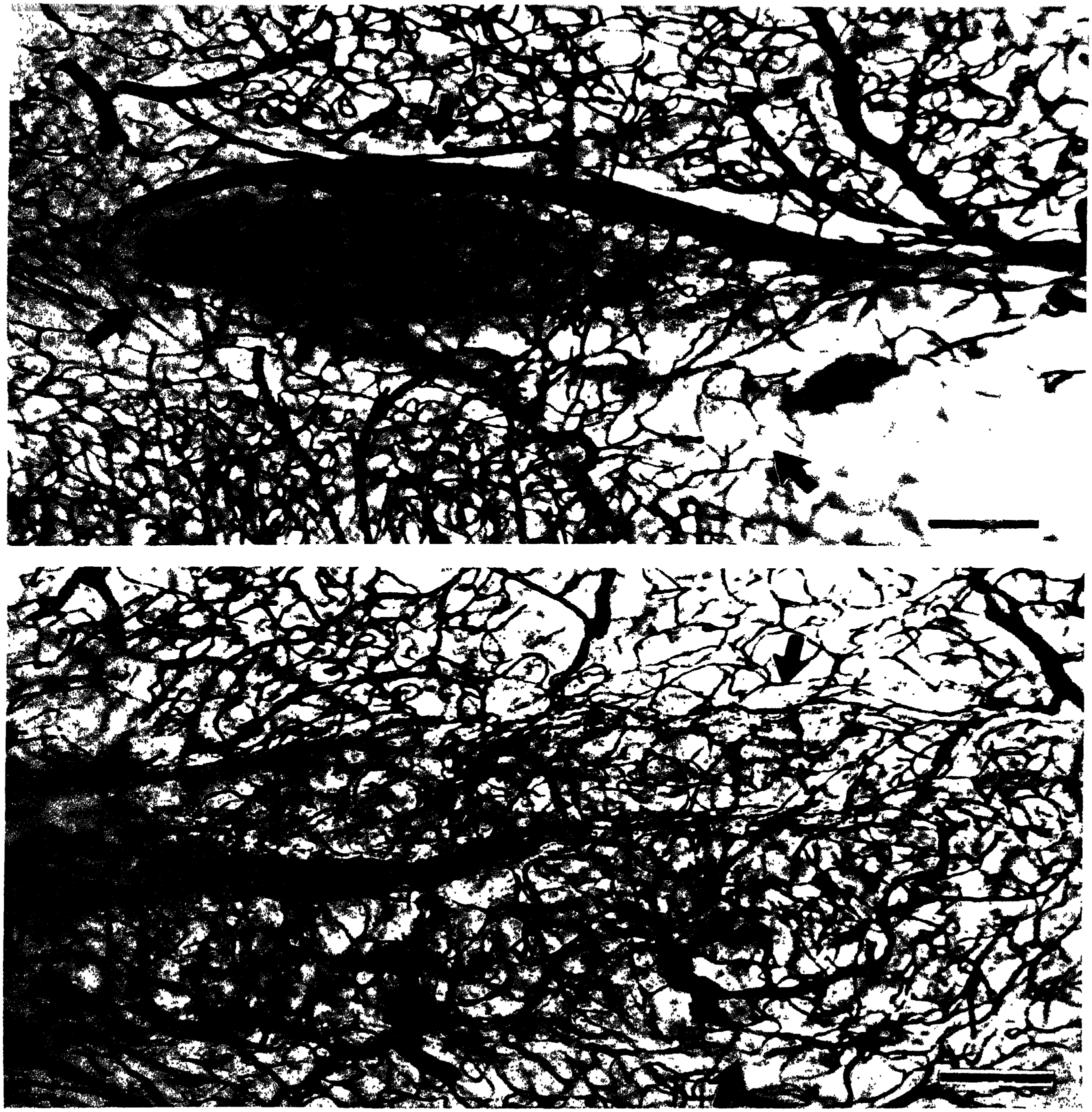


Figure 9 Angioarchitecture of transplanted glio-neuronal aggregates of dissociated neocortical cells from 17-day rat embryo cultivated during 3 days before transplantation. Transcardial Indian ink perfusion. (Scale: 150 μm). **A,** 7 days survival after transplantation. **B,** 21 days survival after transplantation.

Arrows—the border of the transplants located in the striatum of the recipient brain.

fetuses corresponded structurally to the cerebellum of a 19-day-old fetus; for details see Fig 2B, Figs. 7 and 8 show examples of cellular and cytoarchitectural differentiation of transplanted cerebellar and hippocampal fragments, respectively. The most distinguishing features of these transplants were laminar organization of highly differentiated neurons similar to that seen in cerebellum and hippocampus of normal adult rat brains. The donor tissue consisting of cultured fetal brain fragments from different regions completed the process of phenotypic cytogenesis and histogenesis during the initial periods of transplant development within the host brain parenchyma. The other characteristics like high survival rate (90-100%), full anatomical integration with the host brain tissue, formation of fiber connections with the host brain, absence of pathological reactions in the transplantation and in the host brain that define successful transplant (Das 1983) were observed after roller cultivation.

Vascularization and Angioarchitecture of the Transplants: On the first day the transplant was surrounded by blood. The initial signs of capillary ingrowth into transplanted glio-neuronal aggregates were observed by 2-3 day. During the first week the number of fine vessels progressively increased, and at the end of this period the formation of a capillary net was nearly completed (Fig. 9A) The definitive organization of the vascular system within the transplant occurred during the 3rd week. (figure 9B). In some cases the density of the capillary net within the transplant was much higher than in the surrounding host tissue. The pattern of the vascular bed within the transplant could be clearly distinguished from that of the host brain tissue in case of heterotopic transplantation. For example, the radial organization of vessels was seen in transplants of neocortical tissue into the striatum was characterized which normally has a diffuse capillary net. In many preparations it was possible to see a larger vessel located near the border of the transplant giving off fine collaterals into the transplant (figure 9A, B) Angioarchitecture appeared to be determined by the specificity of cytoarchitecture pattern formed during the development of transplanted brain tissue.

Conclusions and Perspectives

The result of the present study and that of the previous data (McLoon et al. 1981, Lindsay Raisman 1984, Brundin et al. 1985, Kamo et al. 1985, 1986, Bernstein & Goldberg 1986, Gibbs et al. 1986, Colombo et al. 1987 and Lindsay et.al. 1987), demonstrate that cultured fetal brain tissue can be successfully used for intracerebral transplantation. However, in contrast to earlier works the present study utilized roller tube cultivation of brain tissue. Reaggregates of dissociated fetal brain cells and fetal brain fragments not only survive transplantation into the adult brain following growth *in vitro*, but also integrate with the host brain and continue to develop without any apparent pathological reaction within either the transplant or the host brain. A particularly significant finding is the ability of the cultured fetal brain fragments to reproduce rigid phenotypic cellular and cytoarchitectural organization after transplantation. Thus, transplantation of fetal brain fragments following suspension roller culture shows distinct advantages over procedures involving the intracerebral grafting of brain tissue explants grown on a solid substrate. We believe that the methodology described here may be applicable to a wide range of neural transplantation experiments and has clinical applications. For example, one of the possibilities suggested by this study would be the transplantation of cultured human fetal substantia nigra into the caudate nucleus of Parkinsonian patients.

Roller suspension cultures of dissociated and reaggregated fetal brain cells undoubtedly have an advantage over monolayer cell culture regarding the methodology of selection of specific nerve cell populations for intracerebral grafting. Roller cultivation also provides possibilities for experiments involving pretreatment of cultured fetal brain tissue with biologically active substances (neuronotrophic and nerve growth factors, antimitotic agents etc.) in an effort to determine their effects on further integration and regeneration processes within the host brain. Experiments of this type are now in progress.

Acknowledgement

The authors are grateful to Dr R E Baker (The Netherlands Institute for Brain Research) for valuable comments on this manuscript.

References

Bernstein J J and Goldberg W J 1986. Transplantation of cultured fetal spinal cord grafts, grown on a histocompatible substrate, into adult spinal cord; *Brain Res.* **377** 403-408

Björklund A and Stenevi U (Eds) 1985 Neural grafting in the mammalian CNS. Fernstrom Foundation Series **5** (Amsterdam: Elsevier)

Brundin P, Barbin G, Isason O, Mallat M, Chanak B, Prochiantz A, Gage F and Björklund A 1985 Survival of intracerebrally grafted rat dopamine neurons previously cultured *in vitro; Neuroscience Lett.* **61** 79-84

Bunge R P, Johnson M J and Thuline D 1983 Spinal cord reconstruction using cultured embryonic spinal cord strips; Spinal Cord Reconstruction. eds C C Kao, R P Bunge, P J Raies, New York: Raven Press 341-358

Colombo J A, Almeida J F and Molina S 1987 *In vitro* culture and labeling of neural cell aggregates followed by transplantation. *Exp. Neurol,* **98** 606-615

Das G D 1983 Neural transplantation in mammalian brain: some conceptual and technical consideration; In; (eds), *Neural Tissue Transplantation Research* R B Wallace, G D Das, (New York) Springer 1-64

Gibbs R B, Pixley S K R and Cotman C W 1986 Transplantation of septal neurons maintained in long-term culture; *Brain Res,* **383** 409-415

Kamo H, Kim S U, McGeer P L and Shin D H 1986 Functional recovery in a rat model of Parkinsonian's disease following transplantation of cultured human sympathetic neurons; *Brain Res,* **391** 372-376

——Kim S U, McGeer P L and Shin D H 1985 Transplantation of cultured fetal human adrenal chromaffin cells to rat brain; *Neurosci Lett.* **57** 43-48

Lindsay R M and Raisman G 1984 An autoradiographic study of neuronal development, vascularization and glial cell migration from hippocampal transplants labeled in intermediate explant culture *Neuroscience* **12** 513-530

——Raisman G and Seely P J 1987; Intracerebral transplantation of cultured neurons after reaggregation in a plasma clot *Neuroscience* **21** 685-698

Mc Loon L K, Mc Loon S C and Lund R D 1981; Cultured embryonic retinae transplanted to the rat brain: differentiation and formation of projections of host superior colliculus; *Brain Res.* **226** 15-31

Paxinos G and Watson C 1982. *The Rat Brain in Stereotaxic Coordinates* (Sydney: Academic Press)

Sladek J R and Gash D M (Eds) 1984 *Neural Transplants: Development and Function;* (New York: Plenum Press)

Victorov I V 1969 The modification of ammonia-silver method of nervous tissue impregnation; *Archives of Pathology (Russ.)* **6** 79-81

——Lyjin A A, Shashkova N A 1985 Cultivation of reaggregated brain cells in high-speed mini-rollers; *Bull Ex Biol Med (USSR)* **98** 632-634

Wallace R B and Das G D (eds) 1983 *Neural Tissue Transplantation Research* (New York: Springer-Verlag)

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 85-94 (1990)

Transplantation of Embryonic Nervous Tissue Stimulates Compensatory-Restorative Processes in the Brain of Mammals with Diffuse Distrophy of Neurons

M A ALEXANDROVA

N.I. Vavilov Institute of General Genetics, USSR Academy of Sciences, Moscow (USSR)

Diffuse dystrophy and death of neurons in the cerebral cortex, hippocampus, and other parts of the brain are characteristic of a number of neurological and neuropsychical human diseases. A model of hypoxic hypoxia was used for simulating morphologically identical degenerative changes in mammals. This model was used to study the possibilities of stimulating the compensatory-restorative processes in the dystrophic neurons by allotransplantation of the embryonic nervous tissue (ENT) in rats. A comprehensive study of the histological, histochemical, autoradiographic, biochemical and physiological parameters was carried out. The following results have been obtained: ENT grafts developed fairly well in the brain of hypoxia-exposed rats; the neurons of transplants formed afferent and efferent links with those of the recipient; there was no rejection or death of the transplants, despite the fact, that they were developing in dystrophic tissue of the recipient, previously exposed to hypoxia, which damages the blood brain barrier (BBB); ENT transplantation stimulated the repair of hypoxia-damaged BBB; prevented dystrophy and death of neurons in the cortex and hippocampus of the recipient and normalized their structure, stimulated protein, RNA and DNA biosynthesis in the affected cortical neurons and normalized the behaviour of such rats. Our results imply that ENT stimulates and normalizes the dystrophic nervous tissue of recipient by providing neurotrophic factors, and also as a result of interneuronal interactions.

Key Words: Transplantation, hypoxia, Regeneration neurons

Introduction

Transplantation of the nervous tissue appears to be one of the most interesting methods for investigating the mechanisms which underlie differentiation, development, and interaction of the CNS cells (Nottebhom 1985). Transplantation of the corresponding embryonic nervous tissue into the brain of neonatal or adult animals make it possible to repair focal damage to the CNS (Bjorklund & Stenevi 1985). The transplanted tissues differentiate into normal nervous and glial elements which remain active and viable for long periods compensating for the nonfunctioning region of the CNS (e.g. models for Parkinson's disease, diabetes insipidus, and hypogonadism) (Wallace 1986).

A number of pathologies affecting the CNS of man result in diffuse dystrophy and death of neurons (Arendt et al. 1983; Bondareff et al. 1982) as seen in Alzheimer's disease (Gash 1987), Schizophrenia, infantile cerebral paralysis and various kinds of hypoxic injuries of the CNS (Gross et al. 1982). Inspite of different etiologies these diseases are characterized by similar morphological manifestations of dystrophic changes in the neurons. To investigate whether regeneration of the dystrophic neurons is feasible by means of the embryonic nervous tissue transplantation (ENT) (Bjorklund & Gage 1986, Shurr & Rigor 1987) a model of hypoxic hopoxia was used (Bogolepov 1979). Hypoxic hypoxia is known to result in severe pathological changes in the CNS. It causes diffuse dystrophy and death of neurons in the brain cortex and hippocampus (figures 1, 2). Hyopoxia was induced by reducing the concentration of oxygen in the inhaled air.

Materials and Methods

Wistar rats were used in all experiments. Adult females, weighing 180 - 200g were used as recipients and 17 - 20 days old rat embryos served as donors. The embryo previously extracted from uterine horns was placed under a stereomicroscope and the brain was extirpated. The embryonic neocortex was sucked into a thin glass needle and injected in the brain of the recipient. Tissue was transplanted into both intact and hypoxic rats.

Hypoxic hypoxia in rats was achieved in a pressure chamber constructed in our laboratory. The atmosphere pressure was reduced to 180 mm Hg during 1 min. Rats were kept under such pressure with a corresponding reduction of oxygen content for 3 min. 30 - 35% of the exposed animals died following clonic spasm. The surviving animals received the transplants. The transplanted animals were sacrificed 4,60,100 and 365 days after the operation. Cross sections containing transplants were obtained from the both the cerebral hemispheres and fixed in 10% formalin. 7mm paraffin sections were stained with cresyl-violet (Nissl technique). Synaptic interneuronal connections between the host and the transplant were studied histochemically utilizing retrograde axonal transport of HRP. For this purpose, 6 months after transplantation, HRP was injected into intact and hypoxia-subjected rats in the transplant or in the adjacent region of the host brain using stereotoxic coordinates 30% solution of HRP with 2% dimethylsulphoxide (0,1-0,2 mkl) was injected for 10 min to animals anaesthetized with nembutal. 33μm frozen coronal secions were prepared as per the technique of Mesulam (1976) with subsequent additional staining with safranin. For estimating DNA, RNA and protein synthesis , autoradiographic and radioisotopic biochemical assay were employed. To elucidate the role of injury, we punctured the brain with a syringe needle in the same region and injected 0,01ml of physiological solution to act as control for all series of experiments. When rats were sacrificed for morphological examination parts of the host hemispheres without transplant were taken for biochemical estimation of brain proteins using polyacrylamide gel electrophoresis. The behavioural experiments were conducted by means of bilateral active avoidance of electric shock in a shuttle box. The permeability of brain blood barrier was investigated by the Coon's immunological method (Coons and Kaplan, 1950). Student T criterion was used for statistical analysis.

Results

The first goal of present work was to elucidate whether it was possible to perform a successful transplantation of the ENT into the brain of rats subjected to hypoxia resulting in pathological changes in the nervous tissue. It was observed that the pathological condition of the recipient's brain did not interfere with the survival and development of the ENT transplants, which developed and differentiated in the same manner as they do under normal conditions (Polezhaev and Alexandrova 1984). The experiments established the feasibility of successful transplantation of tissue from the cerebellum (figure 3), the hippocampus (figure 4) and the cerebral cortex taken from 17-day-old embryos (Alexandrova & Polezhaev 1984). The transplants differentiated and survived fairly well in the pathologically changed brain of the adult recipient. The cortical transplants did not show any pathology even a year after the transplantation into the brain of adult hypoxia-subjected rats (figure 5). Besides the solid pieces of the ENT, the dissociated ENT cells also successfully developed in the recipient's brain (figure 6). Labelling with H^3T, enabled the investigators to identify easily the trasnplanted cells in the recipient's brain. Embryonic cortex cryopreserved for long term was also seen to survive when transplanted (figure 7). In this case the transplanted neurons stained more intensly, while the volume of the transplants was significantly smaller, as compared to those obtained from fresh ENT samples (Polezhaev and Alexandrova,

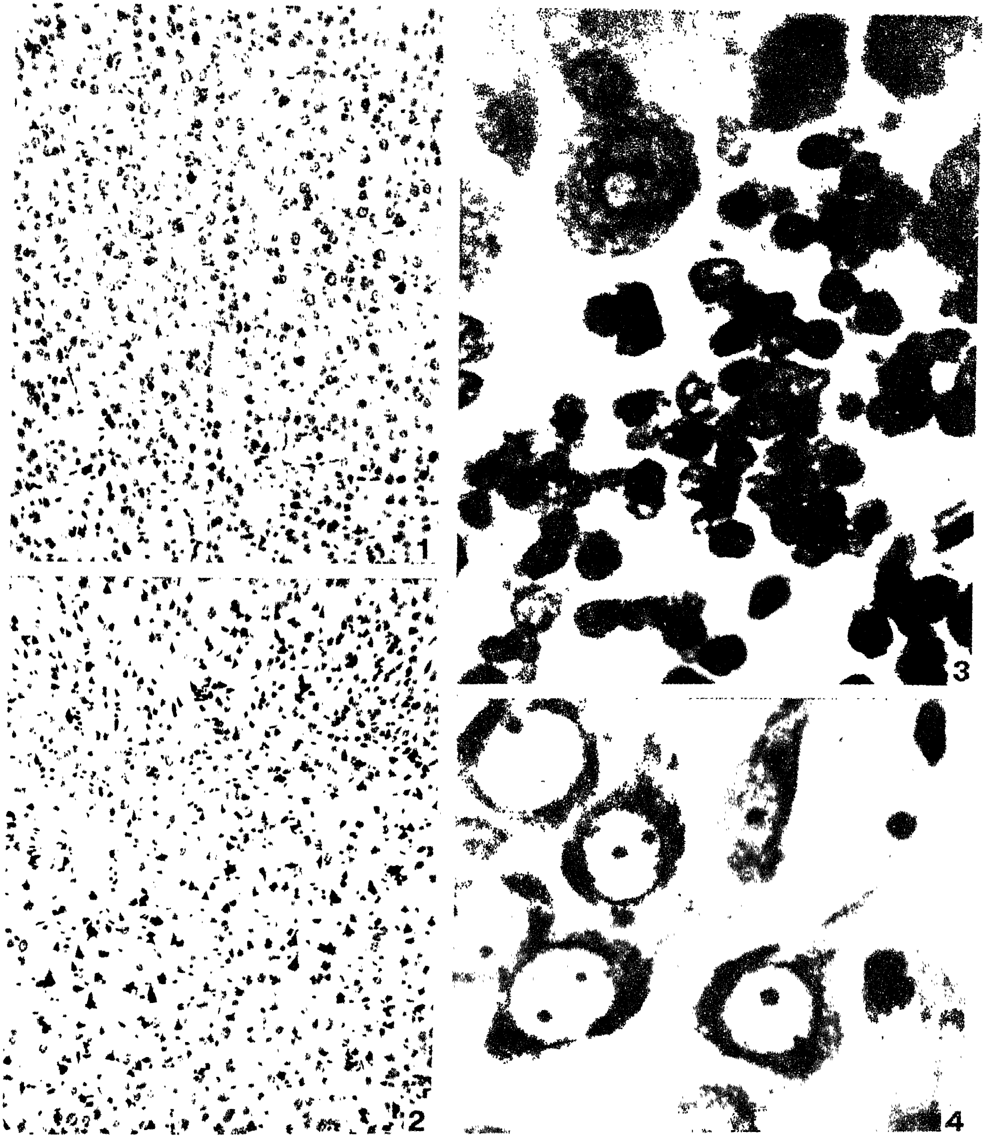


Figure 1-4 Normal neurons in the parietal region of cerebral cortex in rats (Fig. 1) dystrophic neurons in the rat cortex, 60 days after hypoxia treatment (Fig. 2). Cresyl-violet (× 100); Embryonic rat cerebellum transplant in the brain of hypoxia treated rats. Cresyl-violet (× 1000); (Fig. 3) embryonic rat hyppocampus transplant in the hypoxia-treated rat brain. Cresyl-violet (× 200) (Fig. 4)

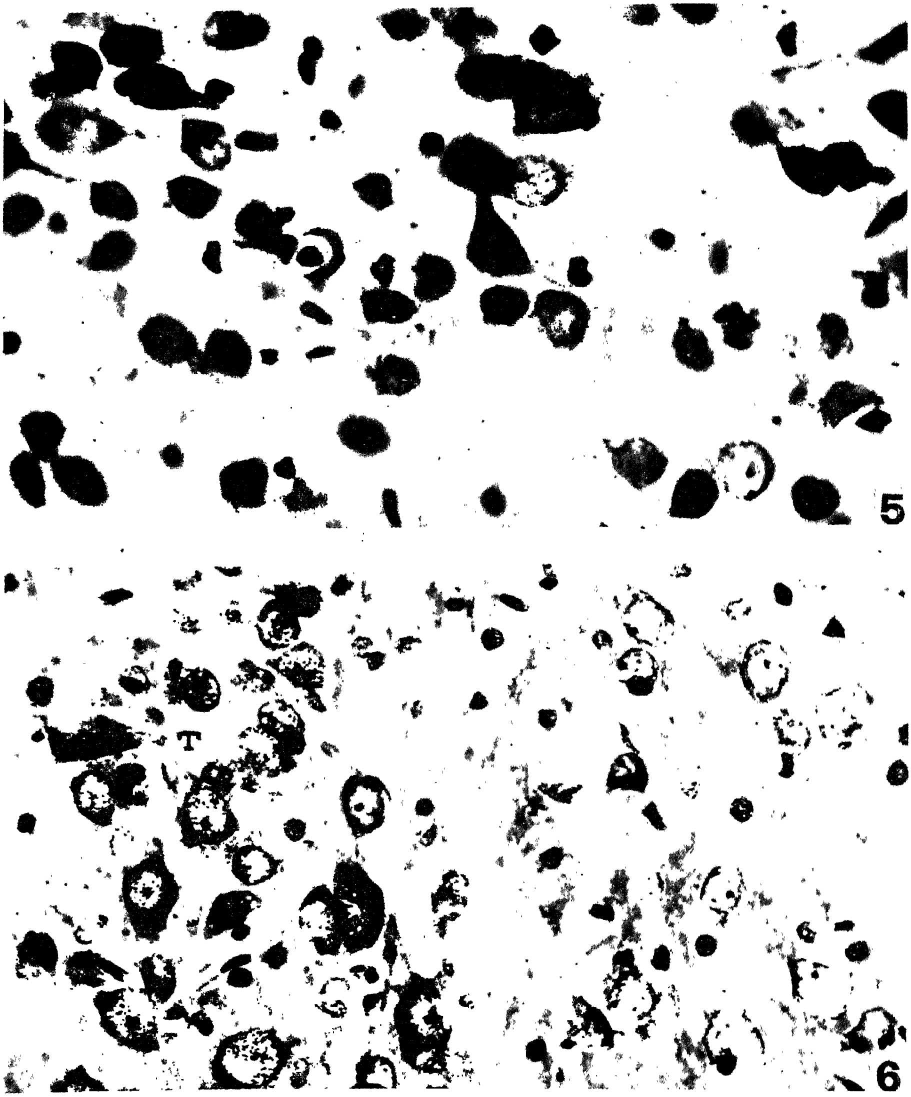


Figure 5-6 5, Embrionic rat cortex transplant following 1 year after the transplantation into the brain of hypoxia-subjected rats. Cresyl-violet (× 200): **6** the dissociated ENT-cells labelled with H^3T (T) successfully developed in the hypoxia treated rat brain. Cresyl-violet (× 400)

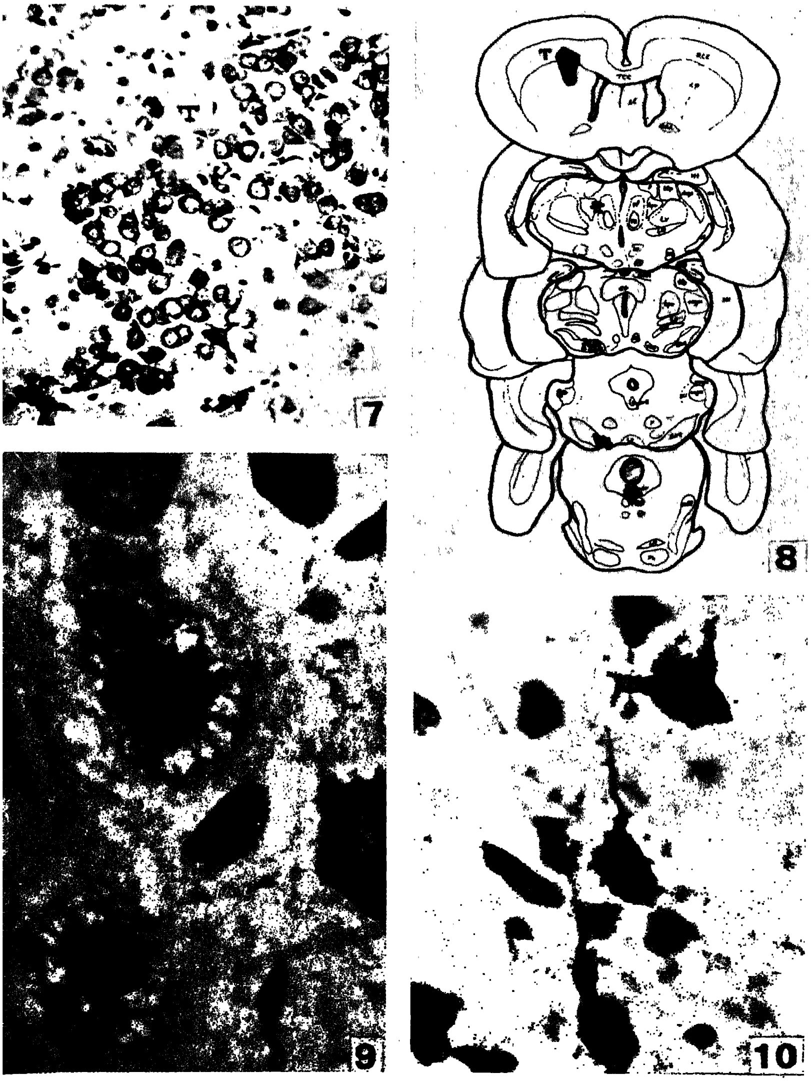


Figure 7-10 Deep frozen ENT cortex cells (T) normally differentiated in the host hypoxia-subjected brain. Cresyl-violet (× 160); (Fig 7),Schematic representation of afferent connection between transplant (T) and neurons of various brain regions of an adult hypoxia treatment rat HRP labelled neurons are marked by large dots;(**Fig. 8**),Vacuolzted neurons in the rat cortex after hypoxia treatment. Cresyl-violet (× 400):(**Fig. 9**),Wrinkled and lysed neurons in the hypoxia-subjected rat cerebral cortex. Cresyl-violet (× 400) (Fig. 10)

1986). Nevertheless these samples differentiated normally and survived over a long period in the brain of the adult recipient. The neurons of the transplant were found to establish active afferent and efferent connections with the recipient's brain as demonstrated by active transport of the horseredish peroxidase (figure 8) Alexandrova 1985. Thus, the ENT transplants can normally survive, differentiate and form interneuronal connections with the neurons of the hypoxia subjected recipient.

What is the effect of hypoxic hypoxia and the subsequent ENT transplantation on the animal's brain? The hypoxia causes diffuse dystrophy of neurons: the reversible dystrophy (RD) is manifested by the vacuolization as well as by the hyper- and hypochromism of the neurons (figure 9); the irreversible dystrophy (ID) is characterized by the formation of wrinkled (figure 10) and lysed cells (Zhabotinsky 1965). Quantitative evaluation revealed that the number of dying neurons of the neocortex constituted 4% in the normal, 36% after hypoxia, and 25% after hypoxia and subsequent ENT transplantation. Simultaneously, a significant decline of density of the neurons was observed after hypoxia treatment, whereas there was an increase in the density of recipient's neurons (Polezhaeve et al. 1986) after the ENT transplantation (figure 11). This suggests that there is an enhancement of the compensatory-restorative processes and preservation of some part of the reversibly dystrophic neurons. The biochemical monitoring of the protein alterations, which goes in parallel to the repair of the nervous cells of hypoxia-subjected rats and also of the rats subjected to hypoxia with subsequent ENT transplantation, has revealed existence of protein fractions involved in the repair processes (figure 12), (Polezhaev et al. 1985). The electrophoresis densitograms of the chromatin proteins show diminuition of the protein synthesis after ENT transplantation. This is further confirmed by autoradiographic and radioscopic biochemical assay of the DNA, protein, and RNA biosynthesis in the neurons and other cortical cells exposed to hypoxia and ENT transplantation (Polezhaev et al. 1988). The transplants of ENT implanted into the dystrophic cerebral cortex of the recipient were found to enhance DNA synthesis

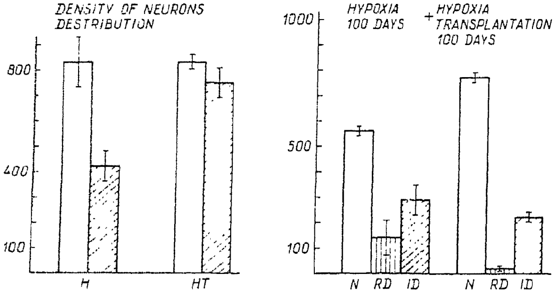


Figure 11 Density of neurons distribution: in normal rat cortex; after hypoxia treatment; after hypoxia with subsequent ENT transplantation.

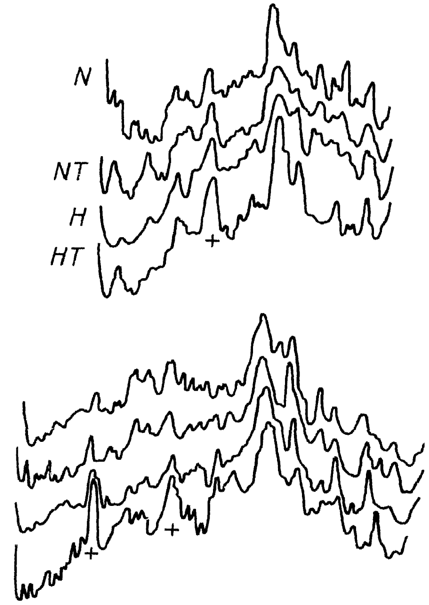


Figure 12 Densitograms of electrophoretic spectra of cytoplasmic neuronal proteins from normal rat cortex (N), rats with transplanted ENT (NT), rats subjected to hypoxia (H), rats subjected to hypoxia with subsequent ENT transplantation (HT) **(a)**; the results of similar experiments with the whole cerebral cortical tissue **(b)**.

mainly in the non-nervous cells. The H^3T label was observed in relation to the small cell nuclei in the endothelium of the vessels, and in discrete not very large neurons. Transplantation, of cerebal cortex promotes the DNA synthesis in 27% of cells, whereas in the hypoxia-affected brain 9% of cells. The autoradiographic data are in good agreement with the biochemical assay, both showed enhancement of the DNA synthesis in the cerebral cortex 4 days after the ENT transplantation (figure 13). Further, autoradiographic investigations have shown that hypoxia results in the inhibition of the protein synthesis in the neurons; the synthesis is normalized after the ENT transplantation (figure 14). The radioisotope biochemical assay confirms these observations. Similar results were obtained in relation to RNA synthesis too (Polezhaev et al. 1988) Both autoradiographic and biochemical data show inhibition of synthesis in the brain cortex after the hypoxia treatment; and its normalization after ENT transplantation (figure 15).

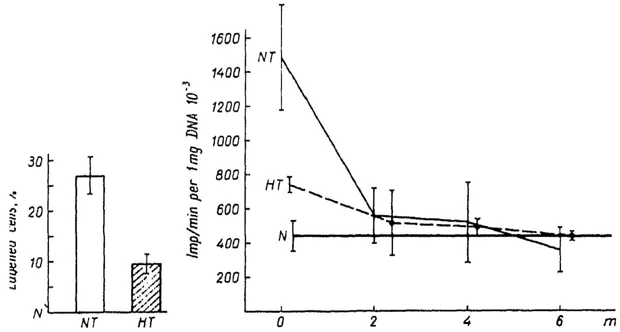


Figure 13 Autoradiographic data and biochemical assaying proved the enhancement of the DNA synthesis in the cerebral cortex detected 4 days after the ENT transplantation

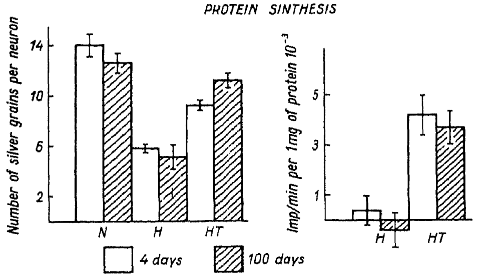


Figure 14 Autoradiographic and biochemical data showing hypoxia results in the inhibition of protein synthesis in the host brain neurons, the protein synthesis normalized after ENT transplantation

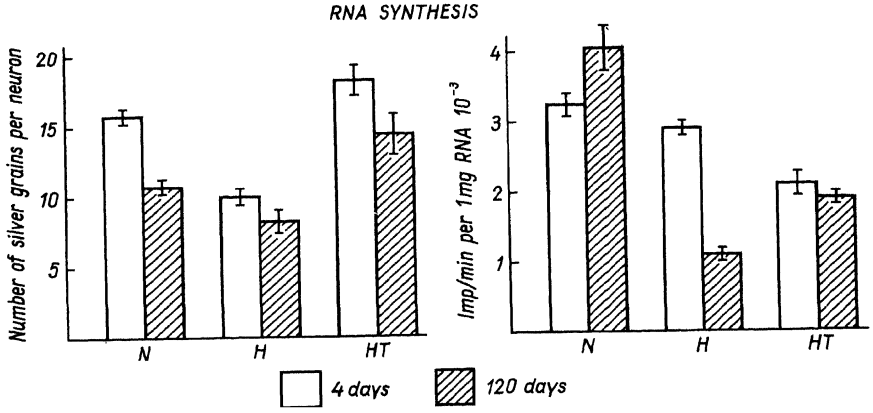


Figure 15 Autoradiographic and biochemical data showing RNA synthesis in the host brain cortex is inhibited after the hypoxia treatment; its significant normalization is observed after the ENT transplantation

Behavioural experiments were conducted by means of bilateral active avoidance of electric shock in a shuttle box. These tests were performed 2.5-3 months following the hypoxia treatment and the ENT transplantation. A comparison of the averaged curves of training revealed significant differences between the groups of normal, hypoxia affected, and hypoxia-ENT transplantation-subjected animals. The hypoxia subjected rats were found to differ greatly from the normal ones in their response to training; whereas the behaviour of the rats subjected to hypoxia and ENT transplantation showed the normal response to training (Figure 16). Normalization of their behaviour was evidenced by comparing the histograms for the latent periods of the conditioned, unconditioned, and intersignal reactions characteristic of these groups of animals. These data confirm the normalizing effect of the ENT transplantation of the rats affected by a diffuse dystrophy of the brain neurons following hypoxia (Girman and Golovina 1988).

The permeability of brain blood barrier (BBB) to the blood immunoglobulins at various time intervals after hypoxia and hypoxia with subsequent ENT transplantation was studied. The permeability of BBB was detected by fluorescence of immunoglobulins in the vessels and tissue of the brain (Coon's technique) Normally the BBB is practically impermeable to immunoglobulins. However after exposure to hypoxia its intactness is found to be damaged and, therefore the fluorescence is observed in the vessels and tissue of the brain. The morphological and quantitative analysis has shown (figure 17) that by the 7th day after hypoxia treatment impairment of BBB reaches its maximum, but later its permeability is gradually restored (Gannushkina et al. 1988). The transplantation of the ENT into a hypoxic brain considerably accelerates the process of the BBB restoration.

Discussion

These investigations have shown that hypoxia causes mass dystrophy and death of the neocortical neurons accompanied by a decline of the DNA, RNA and protein synthesis. It also damages the BBB and results in changes of the animals' behaviour. Our experiments have shown the feasibility of successful ENT transplantation into the brain of rats subjected to hypoxia.

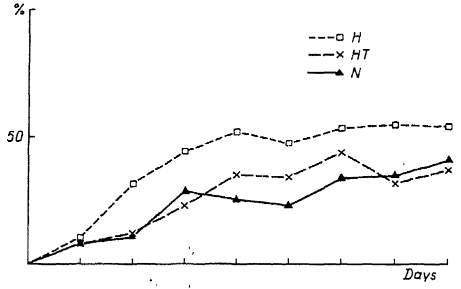


Figure 17 Quantitative analysis showing gradual restoration BBB; the ENT transplantation considerably accelerated the process of the BBB restoration (HT)

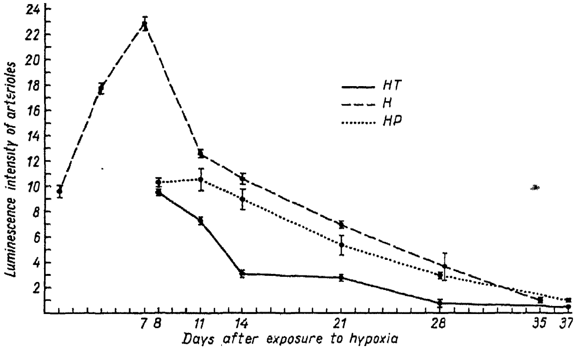


Figure 16 Average curves of training—the hypoxia-subjected rats (H) differ greatly from normal ones (N); whereas the behaviour of rats subjected to hypoxia and ENT transplantation (HT) normalized in the process of training.

The ENT transplantation was found to enhance: 1) regeneration of a considerable part of dystrophic neurons; 2) resumption of the DNA, RNA, and protein biosynthesis in the cells of the damaged brain; 3) normalization of the animals' behaviour; 4) restoration of the BBB permeability.

The ENT transplants stimulate and normalize the dystrophic neurons in the recipient's brain by the following two ways: through the interneuronal connections (Freed & Cannor-Spoor 1988) and by means of neurotrophic agents (Cunningham et al. 1987).

References

Alexandrova M A 1985 Transplantation of embryonic brain tissue into brain of adult rats, intact and exposed to hypoxia; in *Neural Grafting in the Mammals CNS* pp 243-247 ed. A Bjorklund (Amsterdam, New York: Elsevier)

——Polezhaev L V 1984 Transplantation of various regions of embryonic brain tissue into the brain of adult rats; *J Hirnforschung* **25** 89-98

Arendt T, Bigl V, Arendt A, Tennstedt A 1983 Loss of neurons in the nucleus of Meynert in Alzheimer's disease, paralysis agitans, and Korsakoff's disease; *Acta Neuropathol.* **61** 101-108

Bogolepov N N 1979 *The Ultrastructure of Brain in Hypoxia* (Moscow: Meditsina)

Bondareff W, Mount joy C and Roth M 1982 Loss of neurons or origin of the adrenergic projection to cerebral cortex (nucleus locus ceruleus) in senile dementia; *Neurology* **32** 164-168

Bjorklund A and Gage F 1986 Grafting of fetel cholinergic neurons in rat models of aging and dementia; in *Aging* **32** pp 243-258 (New York: Raven Press)

——Stenevi U (eds) 1985 *Neural grafting in the mammalian CNS* (Amsterdam New York: Elsevier)

Coons A and Kaplan M 1950 Localisation of antigen in tissue cells; *J Exp. Med.* **91** 1-13

Cunningham T, Sutilla C and Haun F 1987 Trophic effects of transplants following damage to cerebral cortex; *Ann N Y. Acad. Sci.* **459** 153-168

Das G and Wallace R (eds) 1986 *Neural Transplantation and Regeneration.* (Berlin, New York: Springer-Verlag)

Gannushkina I, Saburina I, Konorova I and Polezhaev L 1988 The role of BBB in embryonal brain tissue transplantation; in *Transplantation of Mammalian Brain Tissue* pp 15-16 eds O Vinogradova (Puschino)

Gash D 1987 Neural transplantation: potential therapy for Alzheimer disease: *J. Neural Transmit. Suppl* **24** 301-308

Girman S and Golovina I 1988 Normalizing influence of nervous embryonic tissue transplant on rat training after hypoxia treatment; *in Transplantation of Mammalian Brain Tissue* pp 19 eds O Vinogradova (Puschino)

Gross J, Lun A and Ihle Odarjuk I 1982 Eienflus von perinataler Hypoxia auf die Entwicklung zellularer und subzellularer Parameter des ZNS: in *Hypoxia und Hypoxiakriterien* **2** pp 68-76 eds G Kramer (Berlin: Humb. Univers)

Freed W and Cannor-Spoor H 1988 Cortical lesione increase reinnervation of the dorsal striatum by substantia nigra grafts; *Brain Res.* **445** 133-143

Mesulam M 1976 The blue reaction product in horseredish neurohistochemistry: incubation parameters and visibility; *J. Histochem. Cytochem.* **24** 1273-1280

Nottebohm F 1985 Neuronal replacement in adulthood; *Ann. N. Y. Acad. Sci.* **475** 143-161

Polezhaev L and Alexandrova M 1984 Transplantation of embryonic brain tissue into the brain of adult rats in hypoxic hypoxia; *J. Hirnforschung* **25** 99-106

—— 1986 *The Transplantation of Brain Tissue in the norm and Pathologi* (Moscow: Nauka)

——, —— and Girman S 1986 Normalization of dystrophic brain cortex neurons after hypoxia and transplantation of embryonic nervous tissue in rats; *J. Hirnforschung* **27** 511-513

——, —— Vitvitsky V, Girman S and Golovina I 1985 Morphological, biochemical and physiological changes in brain nervous tissue after fetel neurons tissue transplantation in adult intact rats and rats after hypoxia; *J. Hirnforschung* **26** 281-289

——, ——, —— Cherkasova L and Girman S 1988 Changes of DNA synthesis in brain cortical cells after transplantation of embryonic nerve tissue into the brain rats after hypoxia; *J. Hirnforschung* 89 (in press)

——, ——, —— Cherkasova L, Saburina I and Girman S 1989 Normalization of protein synthesis in brain cortex of rats after hypoxia by transplantation of embryonic nervous tissue; *J. Hirnforschung* (in press)

—— Saburina, Cherkasova L, Vitvizky V and Timonin A 1988 Normalization of RNA synthesis in the rats brain cortex after hypoxia treatment by means of embryonic nervous tissue transplantation; *Doklady Acad. Sci. USSR* 300 **6** 1477-1481

Shurr A and Rigor B, The mechanism of neural resistance and adaptation to hypoxia; *Letters* **224** 4-9

Zabotinsky J 1965. *Normal and Pathological Morphology of Neuron* (Leningrad: Meditsina)

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 95-100 (1990)

MPTP Model of Parkinson's Disease in Monkey: Implications for Neural Transplantation

G GOPINATH*, A K MAHAPATRA, J C BHARDWAJ, R BANERJEE, A K SHETTY, D N SHARMA and P N TANDON
Neural Transplantation Unit, All India Institute of Medical Sciences, New Delhi 110029 (India)

Total and hemiparkinson primate models were produced by giving intramuscular and unilateral intracarotid injections of 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine (MPTP). The signs such as tremor, rigidity and movement disorders varied despite the uniform dose of the drug given to the experimental animals. Younger monkeys seem to be more susceptible to the drug. Dopaminergic large multipolar neurons showed marked degenerative changes while the non-dopaminergic spindle-shaped neurons were mostly spared. Some of the earlier affected larger neurons appeared to recover later. These observations may be kept in mind while interpreting the results of nigral transplants to the striatum of MPTP-treated primate models.

Key Words: Rhesus monkey, Parkinson model, MPTP, Substantia nigra

Introduction

Parkinson-like syndrome has been produced in primates by various investigators with the view to studying the restorative effect of fetal substantia nigra transplanted into the striatum (Burns et al. 1983, Kit et al. 1986, Bakay et al. 1987, Bankiewicz et al. 1987, and Sladek et al. 1987). Reports on experiments lasting upto 6 months, suggest that the recovery is wholly or partly due to the dopaminergic neurons of the nigral transplant. Because of the varying degrees of recovery in the primate models, Morihisa et al. (1984) are of the view that the recovery is not graft-induced, but spontaneous. The present study was undertaken to correlate the status of the nigral neurons with the behavioural deficits in primate model produced with 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine (MPTP).

Materials and Methods

Six adult rhesus monkeys, weight ranging from 2.5 to 9 kg, were used for producing Parkinson model with the chemical 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine (MPTP). These monkeys had been quarantined after purchase and kept in captivity on standard diet atleast for a period of one year before the experiments.

Three monkeys used for total Parkinson model were given intramuscular injection of MPTP in sterile normal saline at a dose of 0.5 mg/kg body daily for 5 days.

Three monkeys were used for producing hemiparkinsonism because of the difficulty in maintaining the total Parkinson models. Hemiparkinson model was produced by injecting MPTP into the right internal carotid artery. Common carotid artery was exposed in the neck after anesthetizing the animal with Ketlar (10mg/kg body wt). A sterile plastic arterial cannula with a stillet was introduced into the common carotid and guided into the internal carotid artery. The injection of MPTP was done slowly over 5 minutes. The cannula was left *in situ* for 5 minutes more. The dose of MPTP for arterial injection was between 0.5 and 1.6 mg/kg body weight (Bankiewicz et al. 1986).

MPTP after weighing carefully was dissolved in 5 ml sterile normal saline for intracarotid injection and 2 ml normal saline for intramuscular injection. The treatment of individual monkey with MPTP was as follows:

Monkey No. 1776: Young male monkey (1-2 years) weighing 2.1 kg was given daily intramuscular injection of MPTP for 5 days. The total dose was 5.25 mg. Eleven days after the injection the animal was sacrificed.

Monkey No. 1777: Young male monkey (1-2 years) weighing 2.2 kg was administered a total dose of 5.5 mg of MPTP over a period of 5 days. Ventral mesencephalon from a 58-day-old fetus was grafted into the head of the left caudate nucleus on the 9th day after the course of MPTP was completed. On the 16th day after the last MPTP injection and 7 days after transplantation the monkey was sacrificed.

Monkey No. 1805: Young female monkey weighing 3-7 kg (2-3 years) was given 8.6 mg of MPTP intramuscularly over 5 days. On the 10th day after the last MPTP injection, substantia nigra from a 58-day-old fetus was transplanted into the head of the right caudate nucleus. This monkey was sacrificed 108 days after the last MPTP injection.

Monkey No. 1806: Adult female weighing 4 kg (5-6 years) was given intramuscular injection of MPTP for 5 days. Total dose was 8.6 mg. Since this animal had not shown any signs of Parkinsonism for 2 months, it was used for producing hemiparkinson model by injecting 1.6 mg of MPTP into the right internal carotid artery. The animal was sacrificed 60 days after the intracarotid injection.

Monkey No. 1579: Adult male weighing 9.2 kg (over 8 years old) was given a single dose of 3.2 mg. MPTP into the right internal carotid artery. The procedure was repeated with 3.7 mg of MPTP after a month. After 4 days, injection was repeated with 14 mg. On the 18th day of the last injection, substantia nigra from a 55-day-old fetus was grafted into the head of the right caudate nucleus at three different sites. The monkey was sacrificed 4 months after transplantation.

Monkey No. 1812: Adult monkey weighing 8.5 kg (over 8 years) was given 3.4 mg of MPTP into the right internal carotid artery as a single dose. Injection was repeated after 4 days with the same dose but the quantity injected could not be determined due to leakage while injecting. One month after the last injection the animal was sacrificed.

All monkeys were kept under close clinical observations during the period of experimentation.

Transplantation Procedure

Donor tissue: Donor tissue used was substantia nigra obtained from fetuses of dated pregnancy. For dated pregnancy cyclicity of the female monkeys was first determined by daily examination of the vaginal smear. Regularly cycling females were mated with males and sperm positivity in the vaginal swab was determined the next morning. Sperm positive day was kept as zero day of pregnancy. Pregnancy was later confirmed by rectal palpation of the uterus and the uterine artery on the 30th and 40th day postcoitum. Caesarean section of the pregnant monkey was done on the desired date under aseptic conditions after Ketlar anesthesia. After delivering the fetus the wound was closed in layers and the animal was returned to the cage. Adequate antibiotics cover and postpartum care were given to these animals.

The brain of the fetus was rapidly dissected out and immersed in chilled ringer lactate solution. The meninges and blood vessels were carefully removed from the brain. The midbrain was sliced by neat horizontal cuts cranial and caudal to the tectum. There after tectum alongwith a part of the dorsal tegmentum was removed from the ventral midbrain. 8-10 microliters of the ventral midbrain tissue were taken into a glass capillary needle attached to a synringe (Das et al. 1979) for injection into the host striatum. According to the quantity of tissue available 2-3 such injections were given into the host striatum using stereotaxic coordinates. Steretaxic coordinates for larger monkeys were calculated on the heads of autopsy specimens already available in the laboratory.

Host: Two of the total Parkinson models and one of the hemiparkinson models were used for transplantation of fetal substantia nigra into the striatum. Host was anesthetized and the head was shaven and cleaned. Head was fixed on a stereotaxic apparatus after giving adequate support to the chest. The scalp was cut midsagittally and reflected towards either side. The skull was cleaned off the connective tissue and with an electric drill a burr whole was made on the right side using the stereotaxic coordinates for the head of the caudate. The glass capillary needle attached to the syringe was slowly lowered and brought to position in the head of the caudate according to the measurements. The donor tissue was injected by lowering the plunger and the needle was retained in position for two more minutes and then withdrawn.

The monkeys were sacrificed at different time intervals after heavy anesthesia. Monkeys transplanted with substantia nigra were anesthetised and the brain were removed either after fixation with intracardiac perfusion of buffered 4% paraformaldehyde for histology and tyrosine hydroxylase (TH) immunofluorescence or before fixation for monoamine histofluorescenece (Torre 1980). For immufluorescence and monoamine histofluorescene cryostat sections were used. For routine histology paraffin sections were stained with cresyl violet. Numerical density of the neurons in the pars compacta of 3 total Parkinson model and 3 adult control monkeys were determined by semiautomatic method using IBAS Image Analyser.

Results

Monkey No. 1776: Following the third injection, rapid blinking of the eyelids, slowing down of the limb movements and short periods of immobility were observed in the monkey. On the last day of injection monkey assumed a crouching position and remained immobile for long periods of time. Sluggish movements were observed only on prodding. Handling of the food became clumpsy and it could manage self feeding only for 4 more days. Rigidity of the limbs was also noticed. Inspite of oral feeding and intravenous fluid, general condition of the monkey deteriorated. It was sacrificed on the 11th day of the experiment.

Majority of the neurons of the pars compacta of the substantia nigra, when compared to the control, appeared shrunken (figures 1 & 2). Mean diameter in control (3 monkeys) was 43.853 ± 0.546 while in MPTP treated monkeys (3) the measurement was 38.736 ± 2.940. The difference was statistically significant. Nuclei of the neurons appeared cloudy and the margin was indistinct. Many of the cells were hyperchromatic. A few neurons appeared normal, but were smaller in diameter.

Monkey No. 1777: This monkey started showing signs of Parkinsonism* only on the last day of injection. Movements became slow and sluggish and after 2 days it assumed flexed posture and moved only when disturbed. Postural tremors were observed at times. This animal was fed orally with soft fruits and intravenous fluids for 6 days following which it was grafted with fetal substantia nigra. The condition of the monkey continued to deteriorate and it had to be sacrificed on the 21st day.

Neurons of the pars compacta had more or less the same morphology as in the previous monkey. Only very few fluorescing neurons were seen in the compacta region when compared to the large number seen in the control animals (figures 3 & 4). Transplant site in the striatum showed fluorescene for monoamines. However, it was difficult to differentiate individual neurons (figure 5).

Monkey No. 1805: Only on the last day of the injection any kind of disorder in movements was noticed. Rapid blinking of the eyelids, slowing and clumpsiness of the movements were seen. After 2 days the monkey remained stationary in flexed posture. Clumpsy movements were elicited only on prodding. Postural tremors also became apparent. At this stage the monkey was fed on soft fruits orally and fluids intravenously.

On the 10th day after the last injection, substantia nigra from a 58-day-old fetus was grafted into the striatum. On the 6th day after grafting, movements showed improvement and in 2 days monkey was able to transfer food to the mouth. Within the month, movements had improved considerably and the monkey was able to handle food independently. But postural tremors were still present. Suddenly it started deteriorating again and had to be fed orally. In this condition the monkey was maintained for two more months.

Neurons in pars compacta were few in number. Many of them showed chromatolytic changes with eccentrically placed nuclei and clumping of Nissl substance. Some of the spindle-shaped neurons appeared apparently normal (figure 6).

On microscopic examination of the transplanted area of the striatum, a small localised area showed diffuse fluorescence for monoamines. Surrounding area showed red auto fluorescence suggesting tissue necrosis.

Monkey No. 1806: No abnormal signs were noticed in this monkey either during or after the intramuscular injection of MPTP. Following the intracarotid injection of 1.6 mg of MPTP, anticlockwise circling and noticeable rigidity of the left forelimb were noticed. It preferred the right limb for support in the cage. Within the next few days the signs extended to the left limb also. All these signs continued for a month and thereafter there was a gradual recovery. By the end of the second month after intracarotid injection, the monkey appeared normal except for the reduced power in the left forelimb while holding a stick.

Histofluorescence for monoamines in the compacta region of substantia nigra showed a number of fluorescing neurons on the right side, though the density was less compared to the left untreated side. A few chromatolysed and hyperchromatic neurons were seen on routine histology examination. Same features were seen in relation to a few neurons, but much less in number, on the left side.

Monkey No. 1579: Only after the third intracarotid injection signs of motor abnormalities were noticed in this monkey. Flexed frozen position and rapid blinking of the eyelids were noticed after 2 days. Monkey was apathetic and disinterested in food and the surroundings for 2-3 days requiring assisted feeding on soft fruits. After 12 days the monkey started responding to repeated prodding by lunging and open mouth threats Though right limbs appeared normal it preferred to remain in the crouched position most of the time.

At this time substantia nigra from a 55-day-old fetus was transplanted into the striatum. Four days after transplantation food intake increased. Attemps were made to handle food with the left hand without success. But the monkey improved steadily and gradually and became active and started feeding on regular diet within a month. Only very little movement deficit was observed on the left side after two months.

Immunoreactivity for TH on the right side pars compacta of substantia nigra was seen in relation to a few neurons while on the left side many cells were stained positive (figure 7).

The area of the transplant processed for tyrosine hydroxylase had not shown any positive immunoreactivity.

Monkey No. 1812: On the second day after the intracarotid injection the monkey became quiet and listless but no movement disorders were noticed. During this period food intake was also reduced. After 4 days, behaviour of the monkey reverted back to the preinjection status. After the second injection a mild flexion deformity on the left limbs and an occasional anticlockwise circling were noticed for a short while.

Microscopic observation of the pars compacta in this monkey was comparable to the observations in the control monkey.

Discussion

A few significant observations emerge from this study, despite the small number of animals used to produce the Parkinson-like model.

Onset and severity of signs varied from animal to animal belonging to the younger age group treated with identical dose and injection regime of MPTP. Moreover, the younger monkeys appeared to be more sensitive to MPTP when compared to the older age group. Thus, one monkey showed signs after the third injection while the other two started signs only on completion of five injections. This may depend on the fate of conversion of MPTP to MPP (Langston et al. 1984) by monoamine oxidase B. Another interesting observation was the structural details of the neurons in the pars compacta

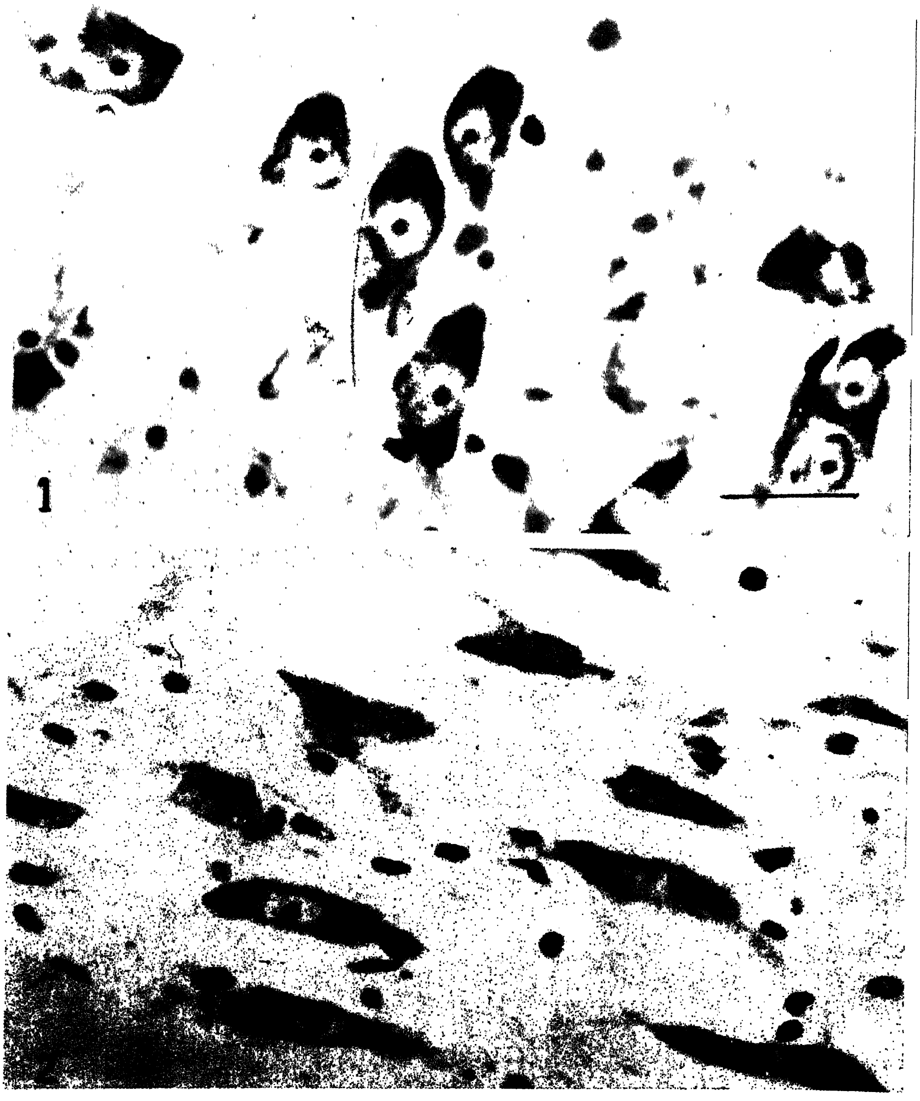


Figure 1·2 Photomicrograph of pars compacta in adult control monkey (Bar = 40μ) Cresyl violet stain; **2:** Pars compacta neurons of monkey, after 3 weeks of MPTP injections. (Bar = 45 M) cresyl violet stain.

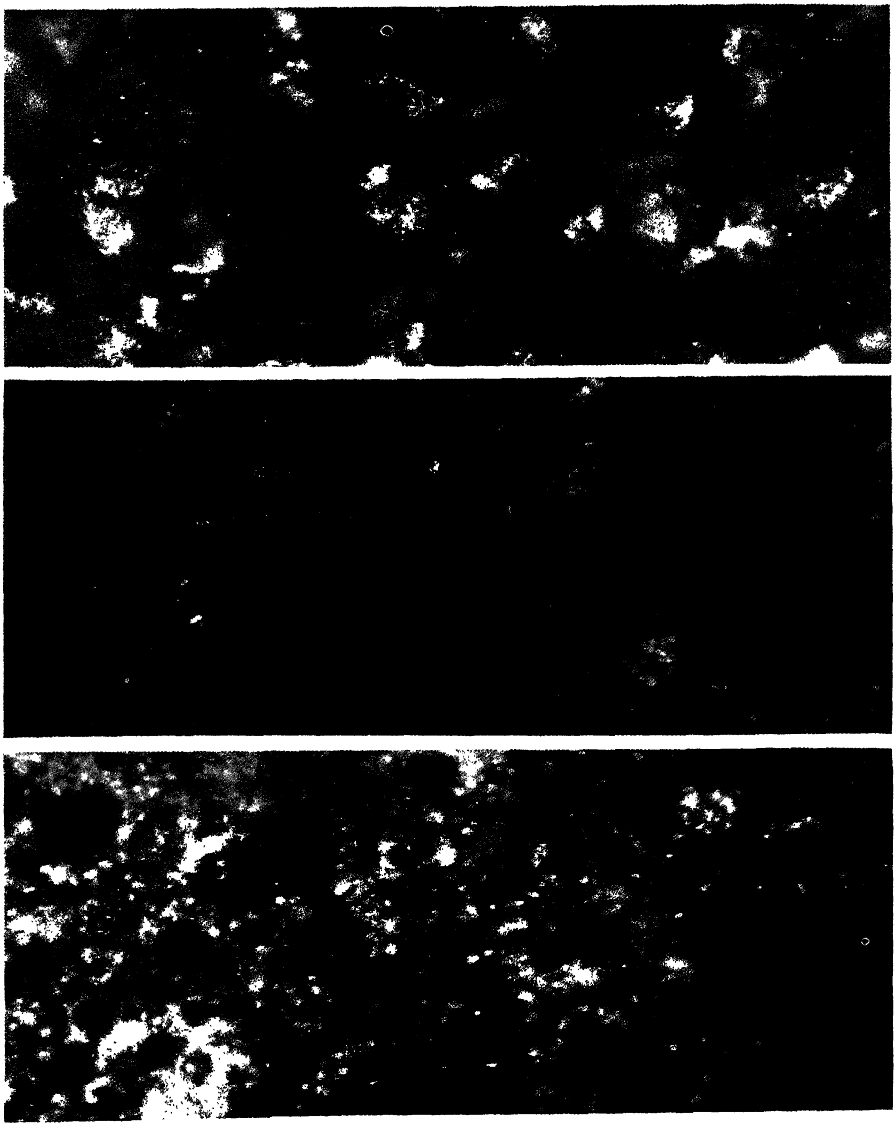


Figure 3-5 Monoaminergic fluorescing cell bodies in pars compacta of normal adult monkey (Bar=45 M) Glyoxylic acid fluoresence; **4**: Monoaminergic fluorescence in the pars compacta of MPTP treated monkey (Bar = 45 M) Glyoxylic acid fluorescence; **5**: Monoaminergic fluorescence of the nigral transplant in the striatum of the MPTP-treated monkey. (Ba = 45 M) Glyoxylic acid fluorescence.

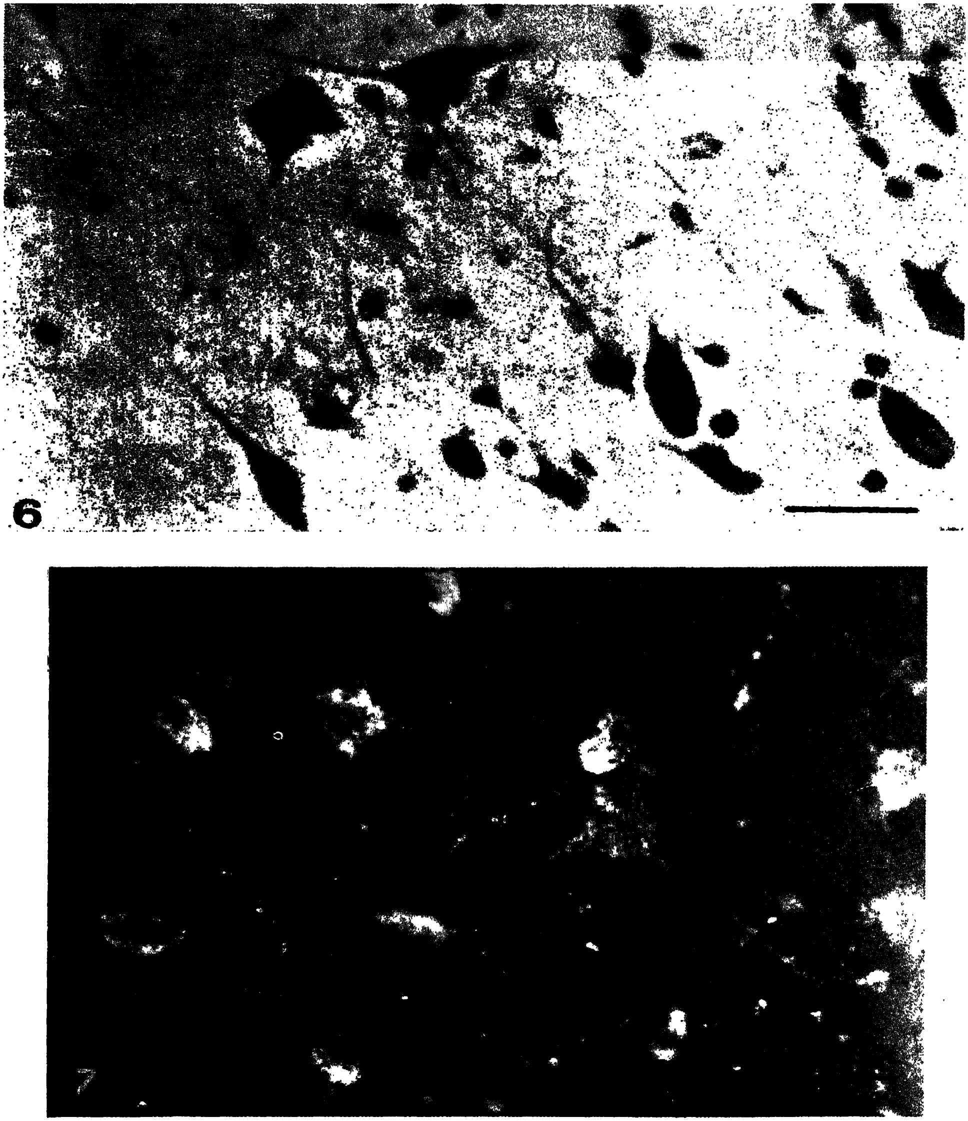


Figure 6-7 Pars compacta showing reduced neuronal density. 108 days after MPTP treatment. Some of the affected large neurons are still retained. Smaller spindle shaped neurons appear normal (Bar = 45 M) Cresyl violet stain; **7**: Tyrosine hydroxylase positive neurons in pars compacta of the contralateral side on the injection showing normal immunoreactivity (40 M)

of the substantia nigra in the three young Parkinson models. Morphological characteristics of the cells more or less are the same in all the three monkeys except for the reduced density of neurons in monkey no. 1805. A few apparently normal neurons are seen in all the monkeys. These observations indicate that neurons are damaged as early as first week after the intramuscular drug administration and all the neurons are not uniformly affected. Mostly the large neurons which are reported to be dopaminergic showed chromatolytic changes. The unaffected neurons are the smaller, spindle-shaped variety, which are non-dopaminergic (Fallon & Loughlin 1985). Though most of the large affected neurons are lost subsequently, it is apparent from monkey no 1805 that some of the affected neurons are retained, which may recover later. This may account for the recovery in monkey no. 1579, which had no surviving dopaminergic neurons in the transplant. Recovery in the immediate post-transplantation period in this monkey could be due to the diffusion of dopamine from the freshly transplanted substantia nigra. In monkey 1805, the recovery during the first month after transplantation appears to be due to the release of dopamine from the degenerating graft, evident from the diffuse fluorescence restricted to a very small area in the transplanted site in this animal.

In our study the older rhesus monkeys appeared more resistant to MPTP toxictity as compared to the younger animals. This is in contrast to the earlier report by Bankiewicz et al (198[illegible]) in *Macaca fascicularis*. According to Bakay et al. (1987), older monkeys already depleted of nigral neurons due to progressive accumulation of pigment, are more sensitive to the insult by MPTP.

In conclusion, younger rhesus monkeys seem to be more sensitive to MPTP toxicity when compared to older animals. The onset and severity of the Parkinson-like signs depend on the effect of MPTP on the substantia nigral neurons which seem to vary from monkey to monkey. Large multipolar neurons which are dopaminerigc appear to be more sentitive to toxicity compared to the nondopaminergic neurons. Some of the dopaminergic neurons seem to recover over a period of time after administration of MPTP. While interpreting the results of substantia nigral transplantation in primate Parkinson model these points should be taken into consideration.

References

Bakay R A E, Barrow D L, Fiandaca M S, Iuvone P M, Schiff A and Collins D C 1987 Biochemical and behavioural correction of MPTP Parkinson-like syndrome by fetal cell transplantation; *Ann. N.Y. Acad. Sci.* **495** 623-641

Bankiewicz K S, Oldfield E H, Chiueh C C, Doppman J L, Jacobowitz D M and Kopin I J 1986 Hemiparkinsonism in monkeys after unilateral internal carotid artery infusion of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); *Life Sci.* **39** 7-16

Bankiewicz K S, Jacobowitz D M, Plunkett R J, Oldfield E H and Kopin I J 1987 Injury induced sprouting into the caudate nucleus, after solid tissue implantation in MPTP-induced Parkinsonian monkeys; *Ann. N.Y. Acad. Sci.* **495** 163

Burns R S, Chiueh C C, Markey S P, Ebert M H, Jacobowitz D M and Kopin I J 1983 A primate model of Parkinsonism: Selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl 1-4-phenyl-1,2,3,6-tetrahydropyridine; *Proc. Natl. Acad. Sci. USA* **80** 4546-4550

Das G D, Hallas B H and Das K G 1979 Transplantation of neural tissues in the brains of laboratory mammals: Technical details and comments; *Experientia* **34** 143-153

Fallon J H and Loughlin S E 1985 Substantia nigra; in *Rat Nervous System* Vol. 1 pp 353-474, (New York: Academic Press)

Kit C A, Cork L C, Eidelberg F, Joh T H and Price D L 1986 Injury of nigral neurons exposed to 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine: A tyrosine hydroxylase immunocytochemical study in monkey; *Neuroscience* **17** 1089-1103

Langston J W, Irwin I, Langston E B and Farno L S 1984 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium in (Mpp+) Identification of a metabolite of MPTP, a toxin selective to the substantia nigra; *Neurosci. Lett.* **48** 87-72

Morihisa J M, Nakamura R K, Freed W J, Mishkin M and Wyat R J 1984 Adrenal medulla grafts survive and exhibi catecholamine-specific fluorescence in the primate brain; *Exp Neurol* **84** 643-653

Redmond D E, Sladek J R Jr., Roth R H, Collier T J, Elsworth J D, Deutch A Y and Haber S 1986 Fetal neuronal grafts in monkeys given methylphenyltetra-hydropridine; *Lancet* 17 **(8490)** 1125-1127

Sladek J R Jr., Collier T J, Elsworth J D, Roth R H and Redmond D E 1987 Effect of fetal substantia nigra transplants on aquisition and performance deficits in MPTP-treated monkeys performing object retrieval/detour task; *Ann. N.Y. Acad. Sci.* **495** 1447

de la Torre J C 1980 An improved approach to histofluorescence using the SPG method for tissue monoamines; *J. Neurosci. Meth.* **3** 1-5

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 101-106 (1990)

Neurotransplant Influences upon Amygdalectomized Rats Behaviour

V P PODACHIN

Institute of Higher Nervous Activity and Neurophysiology, USSR Academy of Sciences, Moscow (USSR)

Therapeutic effect of the embryonic nervous tissue (ENT) is a very well known phenomenon that is why we tried to study the recovery of behaviour of the unilateral and bilateral amygdala lactomized rats.

A group of rats was trained for searching food in one of four boxes. Mechanical lesions were performed in the basolateral part of amygdala on one side. 4-6 days after the operation, ENT of amygdala taken from 18-21 days old embryo was implanted into the cavity. Examination of the food searching behaviour was begun 5-6 days after ENT transplantation. The second group of the animals did not pass through the preliminary procedure of conditioning and was tested only after the operation and transplantation. The third group served as a control and had only unilateral amygdala destruction. The physiological solution was induced in the cavity without ENT.

Significant improvement of food searching bevaviour was observed after ENT transplantation, meanwhile in the control group it never returned to the preoperation level. Since the time elapsed after the transplantation was too short for the development of morphological connections between the transplant and the brain it is suggested that the therapeutic effect was probably due to release of some substance by the transplant or by its influence upon the degree of postoperative inflammation. Influences upon the immunobiological properties of the damaged brain can not be excluded.

The second part of the investigation was performed on the animals with bilateral amygdala lesions. The method of passive avoidance behaviour (PAB) and so-called "conflict situation" (CS) was used. They consisted in avoidance of the dark part of the chamber where the rat had painful electrical punishment or avoidance of the tube where each attempt to drink was also punished. The rest of the procedure was practically the same as in the first experiment with two important exceptions: (i) The experiments started not earlier than 1. 5-2 months after ENT transplantation, (ii) The transplantation was done only into left amygdala cavity.

In conclusion, the degree and phenomenology of the structural changes in the developing nerve cells after partial deafferentation depended on the nature of functional relationships between afferent axons and their target cells.

Key Words: Neurotransplant, Amygalctomized rats, Therapeutic influence

Introduction

Therapeutic influence of transplant on different functions of the damaged brain is already reported by various investigators (Das 1983, Vinogradova 1984, 1988). Their results are based on the data collected for long periods following neuronal embyronic tissue transplantation (NETT). This seems reasonable since the integration between the host and the recipient tissues needs some definite time. On the other hand it is also well known that not all types of behaviour particularly learning can be improved by NETT. Hence proper investigative technique is necessary for evaluating the therapeutic influence of the transplant in the host. The present communication deals with behavioural changes in rat during early and later stages after NETT. The aim of the study is to differentiate the therapeutic influence exerted by NETT during the early phase when there is no obvious integration with the host brain, from that of later period when there is transplant-host integration.

Materials and Methods

Nonlinear rats of about the same weight, sex and age were used for this investigation. The weight varied between 150-200g. Forty-six rats were used to observe early behavioural changes. They were divided into three groups: intact (14), sham-operated (16), experimental with NETT (16). For sham-operation physiological saline was introduced into baso-lateral amygdala area on one side. The transplant (T) was injected in 0.1 mm^3 physiological solution using tuberculin syringe. The NETT was introduced into a cavity in the amygdala region visual cortex or the lower horn of the lateral brain ventricle produced 2-3 days before. The embryonic amygdaloid tissue was collected from 18 - 21 days old rat foetus under nembutal anethesia.

The food searching behaviour was investigated by using a piece of bread soaked with sunflower-seed oil placed in one of the 4 match-boxes in random order. The rats were preliminarily selected by time criterion: if the rat was not active and could not find the bread during 5 min, it was not used for experiments. All rats used for experiments had rather good test behaviour. These experiments started 5-6 days after NETT. In all cases, rats had 24 hr food deprivation.

The second series of experiments was performed in 31 rats which were divided into 3 groups: experimental with electrolytical damage of basolateral amygdala in the bilaterally and NETT soon after. In 11 rats NETT was placed into different brain structures; 10 rats received NETT 10 days after amygdala destruction. The behaviour tested was after a specific type of conditioning, namely: conditioned reflex of passive avoidance (CRPA) which consisted of escape from the dark half of conditional box when the electric current was applied. It meant that if the animals stayed in the dark part of the box immediately they got the electrical shock reinforcement. The second test was the so-called "conflict situation" which meant that each attempt of the animal to drink water from a special arrangement was electrically punished. The degree of T integration with the host brain tissue as well as the different morphological characteristics were studied by Nissl and Golgi methods. The location of T and the extent of brain tissue damage were checked up correspondingly.

Results

The intact rats adapted rapidly to the experimental situation and took only very little time to find food. During the first "pilot" experiments all selected rats spent about 3-4 min for food searching, meanwhile, the trained ones after 10 experiments were able to find food in 1 min and all trials were successful. The sham-operated rats were subdivided into 3 groups: active,

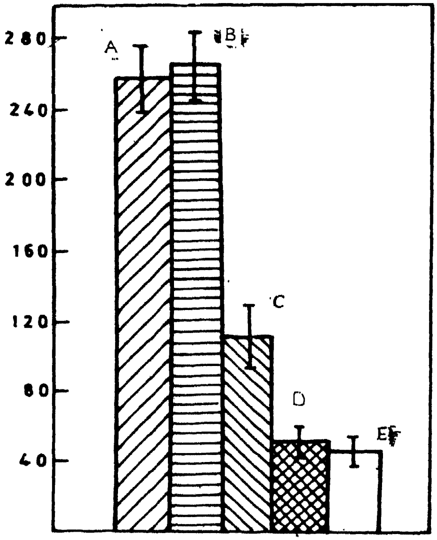


Figure 1 Time of the food search of the rats with:
A —0.1 ml physiological solution injection
B —0.1 ml physiol. sol. + stage specific factor injection (40 mkg)
C —0.1 ml physiol. sol. + serum albuminum injection
D —0.1 ml physiol. sol. + brain embryonic tissue (0.3 - 0.5mm^3) E —intact animals T The averaged data to 14 days

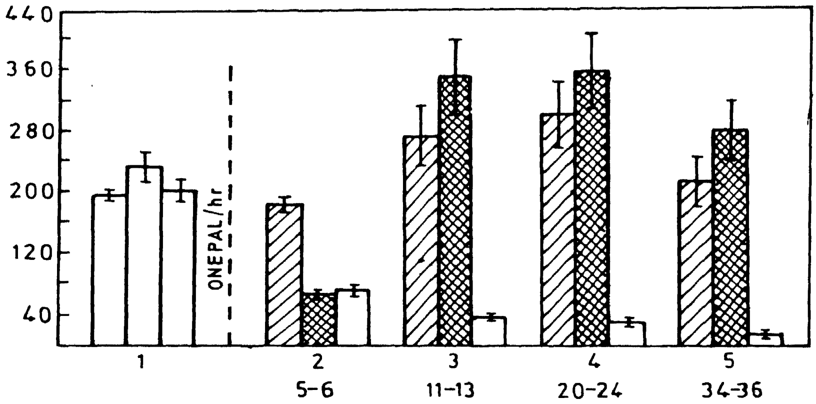


Figure 2 Time of the food search of the rats after unilateral amygdala damage (2 3 days after)
▨ —0.1 ml physiol. sol.
▩ —0.1 ml physiol. sol. + transpl
☐ —intact rats

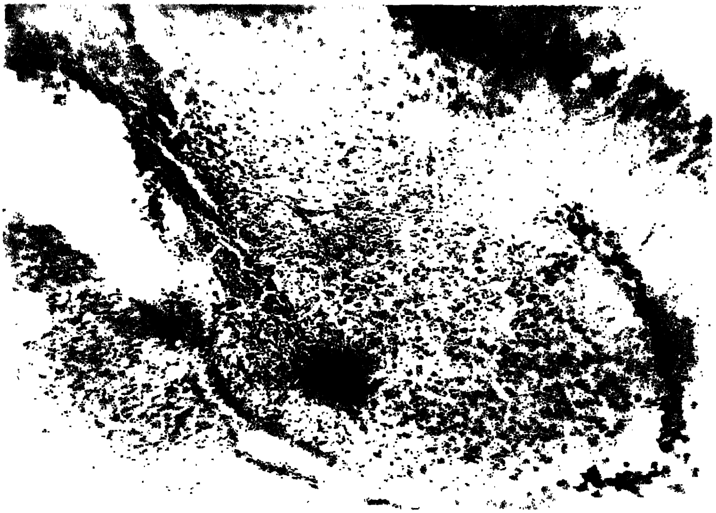


Figure 3 Transplantat integration with host amygdala.

semiactive and passive ones. The locomotion and searching behaviours were very often incomplete and unsuccessful. The difference between the time used for finding the food of the sham-operated and experimental animals was statistically significant ($P < 0.05$). Behavioural changes appeared soon after the amygdala damage and continued usually for 2-4 weeks.

The food-searching behaviour of the rats with NETT into the damaged amygdala did not significantly differ from the intact rats (figure 1), meanwhile the food-searching behaviour of the rats with physiological solution injection two weeks after the operation did not return to the level of the intact rats. Moreover, the use of specific substances such as serum albumin and stage dependent factor did not improve the rats behaviour (figure 1). Since all the mentioned data concern the averaged results two weeks after different lesions, we tried to evaluate the changes which took place at different stages after surgery. Figure 2 shows some results which consequently appeared after the amygdala damage. The most interesting data concerns the behavioural changes 5-6 days after surgery. It was seen that the behavioural parameters of all animal groups improved significantly and were comparable with that of intact rats; meanwhile 2-5 weeks later the behaviour had become worse. The activity of the intact rats became even better than before the operation.

The effect was further confirmed by electric current avoidance test. Practically one punishment was necessary for avoidance behaviour elaboration as it is seen in figure 5. The amygdalectomized rats had very low index of avoidance. In transplanted rats two different kinds of behavioural pattern were noticed. In some rats double effect: in some rats with verified T integration the behaviour was similar to some degree to the intact rats, the avoidance behaviour in another lot became even worse than that of amygdalectomized rats, though the degree of integration as well as morphological features of the neurons were good (figure 6).

It was possible to conclude that NETT had some therapetuic effect at the early stage on the recovery of functions only, whereas later it regressed.

Figure 3 shows some morphological changes of the integrated transplant (T) with the host brain tissue.

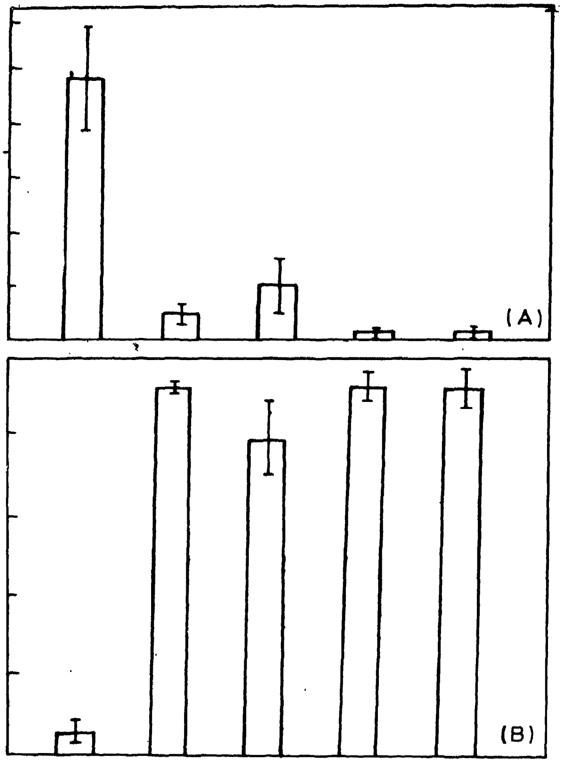


Figure 4 Conditioned reflex passive avoidance behaviour. The upper histogram deals with duration of staying in illuminated part of intact rats, amygdalectomized, amygdalectomized + physiol. sol., amygdalectomized and transplantated, amygdalectomized + tranquillizer (piracetam). The lower histogram shows the same in sequence in the dark part of the box.

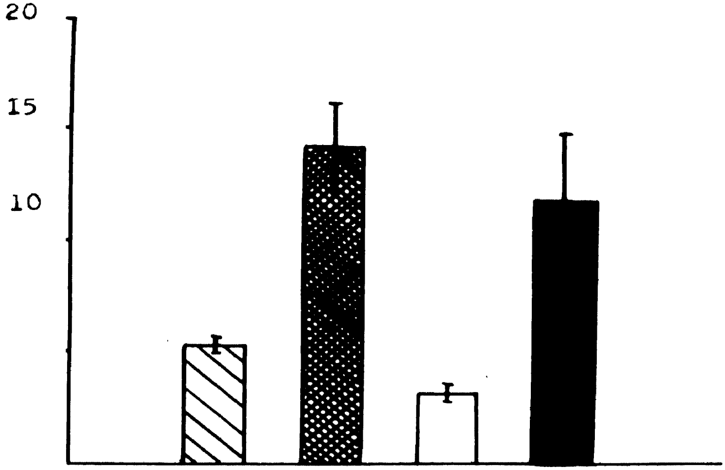


Figure 5 The rats behaviour in conflict situation:
—transplantated rats
—the same
—intact animals
—amygdalectomyzed rats

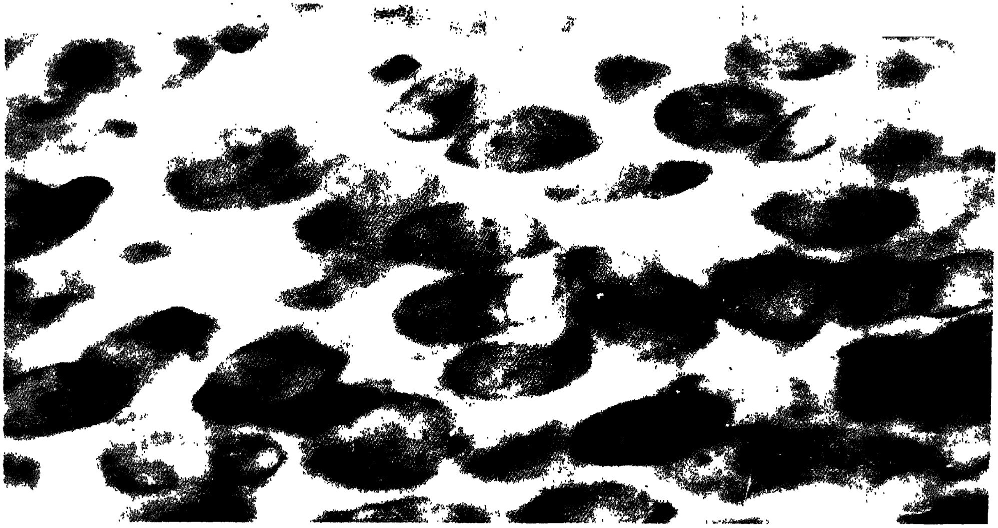


Figure 6 Morphological changes of the transplanted embryonic nervous tissue

The avoidance behaviour (CRPA) elicited soon after the experiments needed several trials. The first (upper) part at figure 4 shows that the control animal preferred to stay in the illuminated part of the conditioned appartment, whereas the amygdalectomized rats preferred the dark part showing complete absence of natural reaction to the illumination. Amygdalectomized rats with injected physiological solution and with NETT also showed same CRPA. The transplanted rats were worse in comparison with simply amygdalectomized rats. Thus, transplantation even as late as 2-2.5 months after surgery had no therapeutic effect.

Discussion

The results of the first series of experiments show transient improvement on the food-searching behaviour for 2 weeks after NETT (Ermakova and Luschekina 1984). According to Vinogradova (1984) NETT may be influencing recovery phenomenon by chemical and morphological means. Release of some chemical substances by transplant seems to be appropriate as stated by Vitvitskii (1984) since the recovery appeared 6-7 days after amygdala destruction and transplant injection. The second possibility is the therapeutic influence of the traumatic procedure as it has been reported by Polejhaev et al. (1985). Nevertheless all these influences are very transient since recovery is observed only for a short period of time. It is possible to conclude that these effects are hardly due to the morphological integration which take place usually around 1.5-2 months after NETT. This conclusion is supported by the present study showing no obvious behaviour improvement by this time. The CRPA changes after NETT showed worsening of the animals behaviour, though the degree of transplant integration with the host brain tissue was high enough. Thus, in this type of behaviour the transplant was powerless to change the response of the animal to painful reinforcement. At the same time the animals behaviour during the conflict situation had improved. Though not all the animals showed the same changes of the behaviour it is possible to conclude that such events reflect the adaptive possibilities of the CNS, since the decreasing fear of punishment permits the animal to reinforce such vitally necessary need as water intake. There are some reasons to think that such behaviour coincides to a certain degree with the adaptive stage of the stress response to the damaging stimuli of the environment (Khonicheva et al. 1988). Probably, the absence of therapeutic influence of transplant is due to inadequate compensation, since the amygdala damage was done bilaterally and NETT unilaterally.

References

Das G D 1983 Neuronal transplantation in mammalian brain: Some conceptual and technical considerations; in *Neural Tissue Transplantation Research* pp. 1-63 ed. B Wallace and G D Das (Springer)

Ermakova I V, Luschekina E A 1984 Neuronal embryonic amygdaloid tissue transplantation influences upon rats food searching behavior; In issue: the young scientists and basic way of biology development. *VINITI* N 6595, Part 2, 76

Galik J, Amori T and Bures J 1985 Electrophysiological investigation of connections between embryonal brain transplants and host cortex (All-Union Symposium "Transpl. Brain Tissue of the Mammalian" Puschino, 14

Khonicheva N M, Luschekina E A, Guljaeva N V, Kurbatova I B 1988 Neural transplantation as a method for the adaptation and memory investigation. (All-Union Symposium "Transpl. of the mammalian brain tissue" Puschino) 65.

Polejhaev L V, Alexandrova I A and Girma S V 1985 The normalization of distrophycally changed brain units of the rats after hypoxia and embryonic nervous tissue transplantation; *Acad. Sci. Report,* 284 1247

Vinogradova O S 1984 Mammalian nervous tissue growth during transplantation into the eye chamber: problems and perspectives; *Ontogenesis* **15** 229

—— 1988 Perspectives and restrictions of neurotransplantations (All-Union Symposium "Transpl. brain tissue of the mammalian brain" Puschino p. 12

Vitvitskii V N 1984 Investigation of the control of the prolipheration processes of the brain units at different stages of growth. The developing brain; *Tbilisi* 50

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 107-108 (1990)

Biology of Tumour Transplant and Fetal Brain Transplant into Adult Rat Brain

S MOHANTY

Section of Neurosurgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005 (India)

Complete fusion between the implanted fetal brain graft and adjacent host brain tissue in many of our experiments did not take place due to excessive neuropile and gliosis at the graft-host junction. On the other hand, a glial tumor transplant showed no such barrier and tumor cells migrated into the host brain.

Key Words: Neural transplantation, Tumor transplant, Invasion, Neuropile

Introduction

Basically both glioma tumor cells and fetal brain are immature CNS cells. Both survive when transplanted into adult rat brain. However, there are fundamental biological difference between the two; the most important being (i) glial tumor cells do not mature whereas immature neurons and glia of fetal brain mature; (ii) neurons establish connectivity through growth of synapses whereas glia participate in the growth of axons; (iii) Glial tumors invade locally whereas immature glia of transplant migrate along fibre tract and blood vessels. Their maturation is characterised by a switch over from vimentin to GFAP expression. A fetal brain graft, on the other hand, is found to establish a limited degree of morphological connectivity without sufficient evidence of lasting functional integrity. The biggest hindrance to migration and establishment of free connectivity is a dense neuropil practically forming a barrier between the fetal graft and the host brain.

Materials and Methods

Tumor Transplant

Nitrosourea-induced F98 rat glioma clone was used. The cultured cells were injected into the cerebral hemispheree of isogenic rats in varying concentrations from 10^5 to 10^2 cells/ml. These grew as a well defined tumor nodule within 7 to 15 days. On histological examination the tumors were mostly anaplastic gliomas. Blood-tumor and blood-brain-barrier was altered as revealed by Evans-blue technique. India ink perfusion revealed vascularization of the tumor.

Fetal Brain Transplant

The embryonic brain (13 day pregnant rat) of 1 to 3 micro litre was used for transplantation in the (cerabrale) hemisphere as per standard technique (Das 1986)

Results

Biological differences of the glial tumor transplant and fetal brain transplant are summarised in table 1. One of the remarkable features in the glial tumor transplant was the free invasion within the host brain. Such a phenomenon of distant migration, although very desirable in the fetal neural transplant for better connectivity and restoration of function, does not occur to any great extent due to dense barrier by neuropile and reactive gliosis. Many of the other characteristics of the tumor transplant are, however, undesirable for a fetal brain transplant.

Table 1: *Biological differences in the glial tumor transplant and fetal brain transplant.*

	Tumor Transplant	Fetal brain transplant
Take-up rate	Excellent	Excellent
Growth	Rapid; kills animals in 2 weeks	slow; animals survive
Invasiveness	+ +	—
Cellularity	+ +	+
Neovascularization	+ +	+
Necrosis	+ +	—
Tumor marker	+	—
Differentiation		
Oedema		

Discussion

Bailey and Cushing (1926) believed that the glial tumors arose from the immature cells of the developing CNS and classified the tumors according to the cell of origin. However, the present findings show that tumor cells undergo genetic change resulting in a complete and radical difference from an immature developing glial cells. Embryonic CNS cells establish physical connection, produce neurotransmitters or neurohormones. Grafting of fetal tissue may be followed by improved function of the animal with experimental forms of neurologic disease or physical injury (Collier et al. 1983, Das et al. 1986).

Developmental studies have suggested existence of regulating mechanisms and trophic factors governing neuronal migration and fibre growth. VIP, a vasodilator in the neocortex, may enhance blood supply to the new grafts; bombesin, a putative growth factor in normal and milignant cells, may be present in fetal transplant as well. Ganglioside GM, thyroxine and hydrocortisone have also been proposed as growth factors in neural transplantation models (Moody et al. 1985, Comissiong 1985). Embryonic retinal fibres transplanted to the acqueduct grew toward the denervated superior colliculus. However, in extensive neuronal loss (dementias and post traumatic conditions), success of neural graft will depend on extensive migration and connectivity in order to restore function. Knowledge of the biology of tumor migration and invasion may help in understanding further the behavior of neural tranplant.

Acknowledgement

The author is grateful to Prof. W Wechsler, Institute of Neuropathology, University of Dusséldorf, FRG, for help of tumor transplant experiment. Neural transplant experiment was part of thesis of Dr I Ahmad under the guidance of the author and Dr S Samal, Department of Anatomy, IMS, BHU.

References

Bailey P and Cushing H 1926 A classification of tumours of the glioma group (Philadelphia: Lippincott)

Collier T J, Gash D M, Sladek J R 1983 Transplantation of fetal noradrenergic neurons into dorsal bundle-lesioned adult rats: effect on habituation, learning and memory; *Soc Neurosci Abstr* **9** 559

Comissiong J W and Toffano G 1985. The effect of GM$_1$, thyroxine and ehydrocortisone on fetal catcholergic nuclei transplanted into the transected spinal cord of the adult rat; *Soc. Neurosci. Abstr.*111255

Das G 1986 Neural transplantation in spinal cord under different condition of lesions and their functional significance; In *Progr. in life Sci.* pp 1-61 eds Gopal D Das and R B Wallace (Newyork: Springer-Verlag)

Moody T W, Getz R L, Saffer M M, Rosenstein J M 1985. Neuropeptide receptors are present in fetal neocortical transplants; *Soc Neurosi Abstr* **11** 603

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 109-124 (1990)

Perspectives and Limitations of Neurotransplantation

O S VINOGRADOVA
Institute of Biological Physics, USSR Academy of Sciences, Puschino-on-Oka (USSR)

While the therapeutic application of neurotransplantation has gained great momentum all-over the world, its theoretical bases are still not well understood. One of the most important problems is the limit of the brain immunological privilege. A number of complex factors provide protection to the brain against the immune system. Nevertheless, this protection, might be overcome by a set of specific mechanisms. Experiments demonstrate lower survival rate of xenografts vs. allografts and their vulnerability to minor disturbances of the blood-brain barrier. Low histocompatibility of donors and recipients may lead not only to the limitation of growth and differentiation of the embryonal neurons, but also to dramatic changes in their organization. Thus, hippocampal anlage grafted to the hosts of the same strain develop well-organized pyramidal layer; this does not occur if the recipient belongs to another inbred strain. Evaluation of the compensatory influence of brain grafts upon deranged nervous functions shows that a number of quite different phenomena are responsible for it. First, there is initial non-specific trophic influence of the grafted embryonal tissue upon the lesioned brain, resulting in prolonged survival of the lesioned host's neurons which deprived of their normal targets or inputs, would otherwise degenerate. The second, more specific, is the effect of the grafts of biochemically specialized brain tissue, e.g. producing dopamine, noradrenaline, serotonin or acetylcholine. In this case the graft may act as an endogenous source of a neurotransmitter deficient in the host brain. Specificity of such compensatory effects upon various behavioural and biochemical parameters was demonstrated in our experiments on transplantation of locus coeruleus and midbrain raphe tissue after specific neurotoxic lesions of the brain.

Key Words: Neurotransplantation, Immunology, Limitations, Perspectives

Introduction

Inspite of the fact that the first attempts in neurografting were done more than a century ago (Thompson 1890), the few papers in this field did not attract much attention of the scientists. The new interest in neurografting in the 70-s, with application of new methods of investigation (Das & Altman 1972, Olson & Seiger 1972, Bjorklund & Stenevi 1979), showed its great potentials for both basic and applied investigations of the brain. By now neurografting has proved to be a very powerful method of investigation of such important problems as ontogenesis of the brain, regeneration of the lesioned nervous tissue and possibility of compensation of the brain functions. Moreover, the clinical application of the neural tissue grafting has been started in several countries of the world, though the theoretical basis of this therapeutical approach are still far from clear. This increases the responsibility of basic scientist even greater. Hence, it is necessary to critically evaluate not only achievements but also the controversial problems and possible limitations of neural transplantation.

This paper analyses and illustrates two of these problems:

(i) Immunological privilege of the brain and eye versus its limitations

(ii) Specificity versus non-specificity of compensation of the brain functions following neurografting.

Limitations of the Immunological Privilege.

Transplantation of the nervous tissue and its long-term survival is possible because of so-called immunological privilege of the brain and eye (Medawar 1948). By now it is shown that it depends upon a variety of complex factors:

(i) Absence of an independent lymphatic drainage excludes the usual route of development of immune response

(ii) Presence of the blood-brain barrier prevents contact between the cellular elements and antibodies circulating in the blood. The grafted neural tissue induces the development of typical barrier capillaries (Steward & Wiley 1981, Zhuravleva et al. 1984)

(iii) The antigen-presenting cells are absent from the brain parenchyma (Head & Billingham 1985)

(iv) The brain cells (neurons, astrocytes, oligodendrocytes) normally do not express class I and II antigens of major histocompatibility complex (Lisak et al. 1983, Wong et al. 1984, Hickey & Kimura 1987)

(v) The nerve cells have late expression of the tissue-specific antigens, and thus the grafted embryonic nervous tissue might be "undetectable" by the immune system of the recipient (Matsuko & Shimada 1983, Bauer & Hurtenbach 1986)

(vi) There are also some additional factors, e.g. Similarity between some surface antigens (Thy-1, Leu-7) of the nervous cells and T-killers (Schuller-Petrovic et al. 1983). As T-killers are not cytotoxic to oneself (Young & Liu 1988), the "false recognition of the nervous cells might help to protect.

(vii) Introduction of an antigen into the brain and eye might induce tolerance (induction of T-suppressors) instead of immune response of rejection ("deviation of immunity") (Kaplan & Streilein 1977)

(viii) Biogenic amines have strong immunosuppresive effects (Walker & Codd 1985, Frohman et al. 1988), widespread innervation of the brain by aminergic neurons may participate in suppression of local immune responses

All these factors permit transplantation of the nervous tissue between histoincompatible organisms, even between the animals of different species. Nevertheless, the exprimental results on xenotransplantation are very contradictory. While some authors demonstrated the possibility of survival and development of xenografts without immunosuppression (Daniloff et al. 1985, Kamo et al. 1986, Zimmer et al. 1988), the others claimed that survival of a xenograft is possible only under immunosuppression with cyclosporin A, or in genetically immunocompromised hosts (Armant & Turner 1988, Bickford-Wimer et al. 1987, Inoue et al. 1985).

In experiments by Kitchigina in our laboratory, hippocampal tissue of the embryonal (E18-19) Wistar rats was grafted into a small acute cavity in the septum of adult Chinchilla rabbits after complete transection of connections between the host's septum and hippocampus without any immunosuppression. This model was chosen for tracing the functional connections between the host septum and the grafted hippocampus. As it is established that prominent rhythmic theta-modulation, expressed in EEG and cellular activity of the hippocampus primarily depends upon the integrity of the sept-hippocampal connections (Rawlins et al. 1979, Vinogradova et al. 1980) the appearence of theta-modulation in the grafted hipocampal neurons can be used as an additional indication of the establishment of functional brain/graft connections. Extracellular investigation of the grafted neurons was performed in chronic conditions, in unanaesthetized and slightly restrained rabbits. Registration of neuronal activity started 7-8 weeks after the grafting and continued for 7-10 days. Some of the hosts (control) were not subjected to microelectrode studies. All grafted animals were sacreficed 9-10 weeks after the grafting. Nissl stain of the brain tissue was used.

Electrophysiological investigations revealed that the neurons of the hippocampal xenografts possessed spontaneous activity of two types. The majority of the cells

(75%) had low-frequency discharges (mean±SE = 4.8±2.0 sp. s⁻¹) with so-called complex spikes (dense bursts of 3—5 spikes with decreasing amplitude). A smaller group of cells (25%) had high-frequency activity (25±1.9 sp. s⁻¹) without complex spikes, but with short periods of unstable rhythmic modulation in theta-range (4-6 Hz) (figure 1). These characteristics of neuronal activity exactly correspond to those described in the normal rat hippocampus (Fox & Ranch 1981, Foster et al. 1987). The expression and stability of the theta-modulation of the grafted neurons increased as a result of various treatments of the host animal. Injection of physostigmine (0.1 mg (kg, i.v.) which is known to increase influences of the rhythmically discharging septal cholinergic neurons upon the hippocampus and thus to evoke stable theta-rhythm in it, increased the number of theta-modulated grafted neurons and stability of such modulation (figure 2). The same effect was produced, as in the hippocampus *in situ)*, by electrical stimulation of the host brain (midbrain reticular formation and ventral part of diagonal band) and by sensory stimuli of various modalities. Besides, electrical stimulation of the adjacent areas (e.g. lateral septum of the host brain) evoked orto-and antidromic responses of the grafted neurons (figure 3). Sustained tonic excitatory or inhibitory reactions and on-off effects to sensory stimulation of the host animal were observed in 58% of the grafted neurons (figure 4). These reactions also closely resembled effects of sensory stimuli in the normal hippocampus (Vinogradova 1975).

Nevertheless, rapid deterioration of the neuronal activity was detected during chronic experiments. After 5 to 7 days of repeated microelectrode penetrations the number of active units and their responsiveness to electrical and sensory stimulation of the host brain decreased and abnormal types of activity (easy depolarization of the cells, epileptiform discharges) appeared.

Histological analysis reveald that the surviving xenografts, which were not subjected to electrophysiological investigation, did not have any signs of lymphocytic infiltration. The majority of the surviving grafts significantly increased in their volume (600 to 800% of initial value). Typical pyramidal neurons were present in the grafted hippocampus, though their organization into a typical layer was absent. Areas of direct confluence of the tissues without glial scar were present (figure 5A). This was persumably true also for the electrophysiologically tested grafts, but all of them

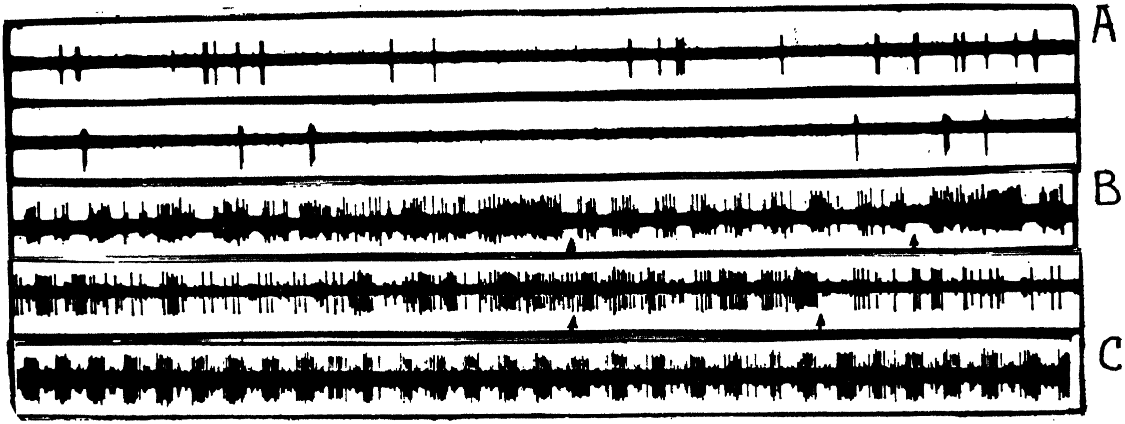


Figure 1 Spontaneous neuronal activity of the hippocampal xenografts. **A**, Low-frequency discharges with complex spikes, **B**, High-frequency discharges, sometimes with weak periodic rhythmic theta modulation (indicated by arrows); **C**, An example of typical neuronal activity with stable rhythmic theta-bursts recorded from the host septum below the graft, (Time calibration 500 ms)

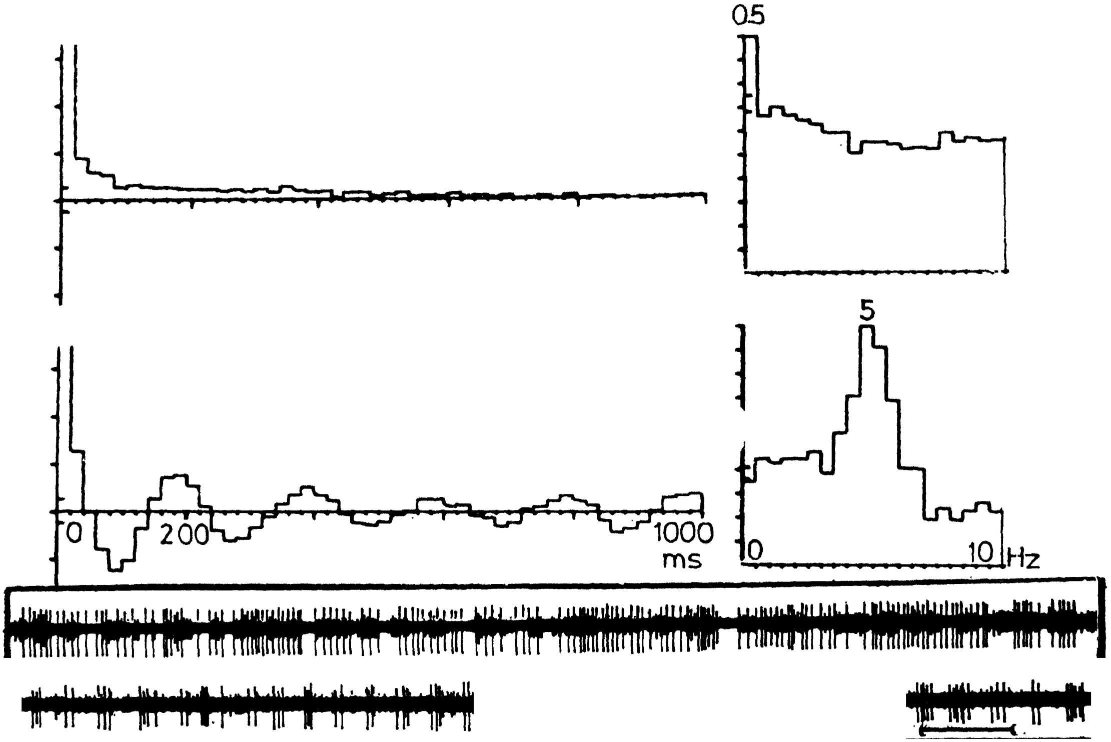


Figure 2 Effect of physostigmine on the neurons of the hippocampal xenograft. Autocorrelation histograms (left, bin 20 ms, period ls) and histograms of spectral densities (right, bin 0.5 Hz) of neuronal activity before (above) and after injection of physestigmine. Stable rhythmic modulation at 5 Hz appears Examples of neuronal activity before and after injection of physostigmine are shown below. (Time 500 ms)

were heavily infiltrated by lymphocytes. Neurons with relatively normal structure were dispersed between the degenerating cells. The immune response was especially strong in the grafts with maximal periods of investigation (8-10 days); their tissue was partly necrotic (figure 5B).

Thus, the xenografted nervous tissue can survive, grow and differentiate. Moreover, it can express morphological and functional organotypic features. Effective connections develop between the host brain and xenograft, and chimeric synapses (i.e., in which presynapse belongs to rabbit, and postsynaps—to rat) can transmit sensory information. Nevertheless, the existence of the xenografts is precarious; any influence which deranges the blood-brain barrier or activates the immune system might be fatal for their survival. We

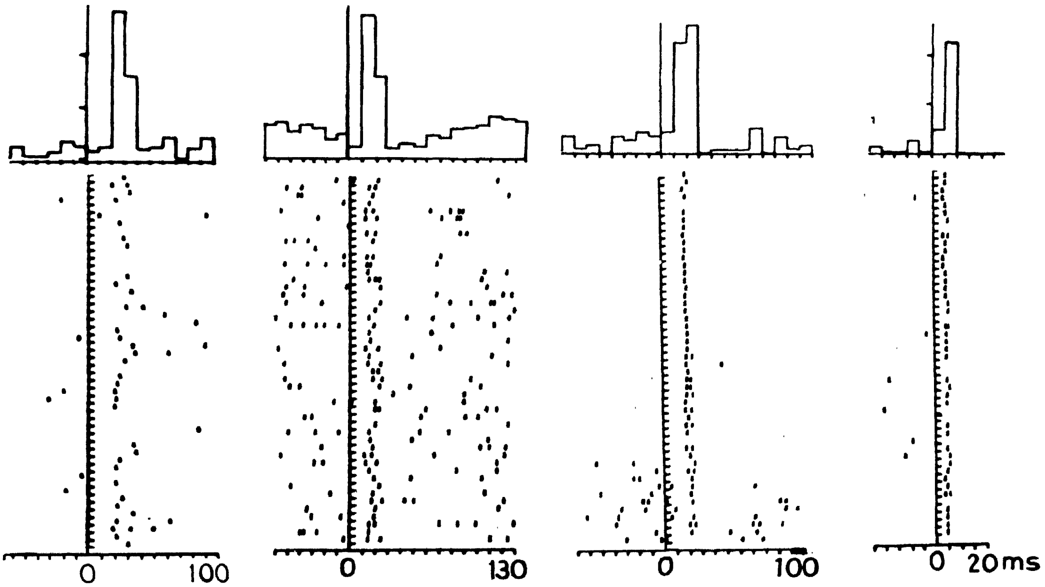


Figure 3 Responses of the neurons in the xenograft to electrical stimulation of the host's lateral septal nucleus. Averaged peristimulus time histograms and raster dot displays are presented. (Time bin is 10 ms, exept the last histogram, where time bin is 5ms (presumably an antidromic response)

suggest that in our experiments, mechanical lesion of the capillary wall by penetrating microelectrodes made the grafts vulnerable to the intrusion of the immune system. The rapid development of rejection of the grafted tissue indicates potential development of the afferent arc of the immune response against it (Rao et al. 1987).

The minor genetic differences between the donor and host may result not in straightforward rejection of the graft, but in more subtle defects of its growth and development. This is illustrated by investigation by Saifullina in our laboratory. The same tissue. embryonal hippocampus of the Wistar rats was used for the grafting. But in this case it was transplanted into the anterior eye chamber (AEC) of adult rats which belonged to Wistar strain (the same as donors) and to WAG inbred strain (derived from Wistar, but genetically not identical to it). Intravital transcorneal morphometric investigation of the grafts' development was performed for three months after the grafting into the AEC. After that histological quantitative analysis of Cresyl Violet-stained grafts was done. Omitting the details, only the main results are summarized here.

In all Wistar recipients (n = 34), exept two, the hippocampal tissue significantly increased in volume (600 - 800% of initial value) and attained organotypic elongated shape ("mini-hippocampus"). This correlated with organization of the pyramidal neurons into well-delineated layer with easily recognizable CA and CA_3 hippocampal subfields. The growth of the grafts in the AEC of WAG hosts (n = 19), was more limited (200 - 400%), and the majority of the grafts were of

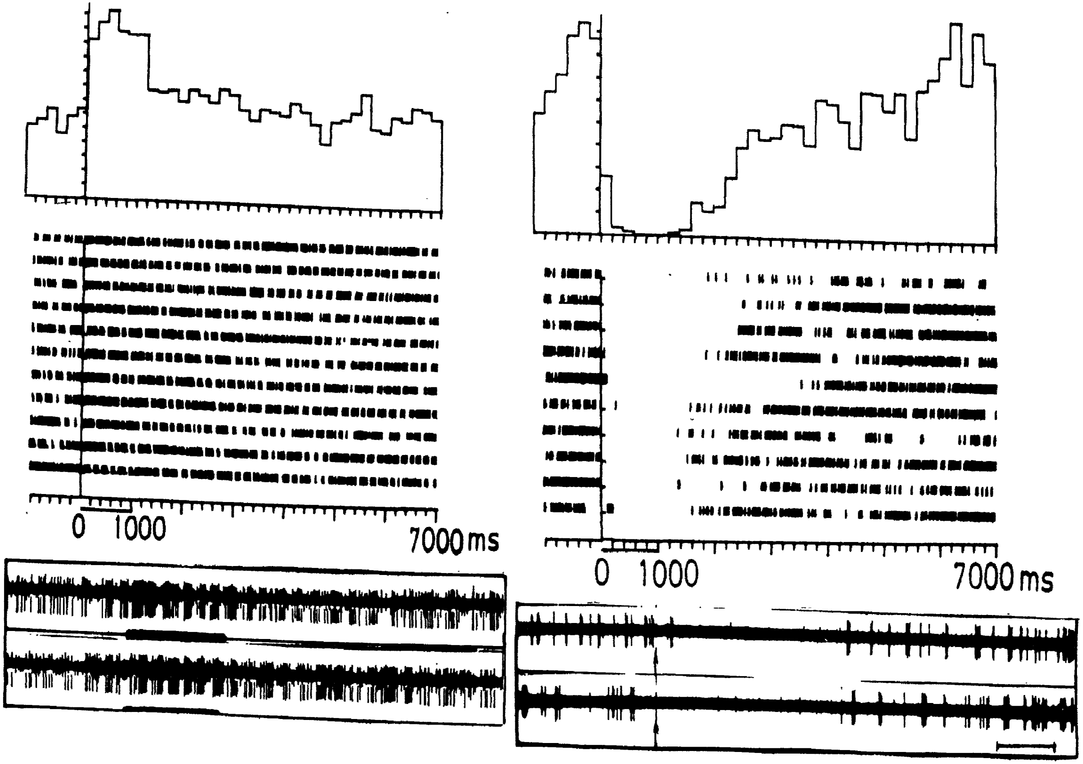


Figure 4 Sustained tonic excitatory (left) and inhibitory (right) responses of the grafted cells to sensory stimulation (pure tone and air puff) of the host animal. Peristimulus time histograms (bin-200 ms) and raster dot displays are shown. Examples of recorded activity are shown below. (Time calibration 500 ms)

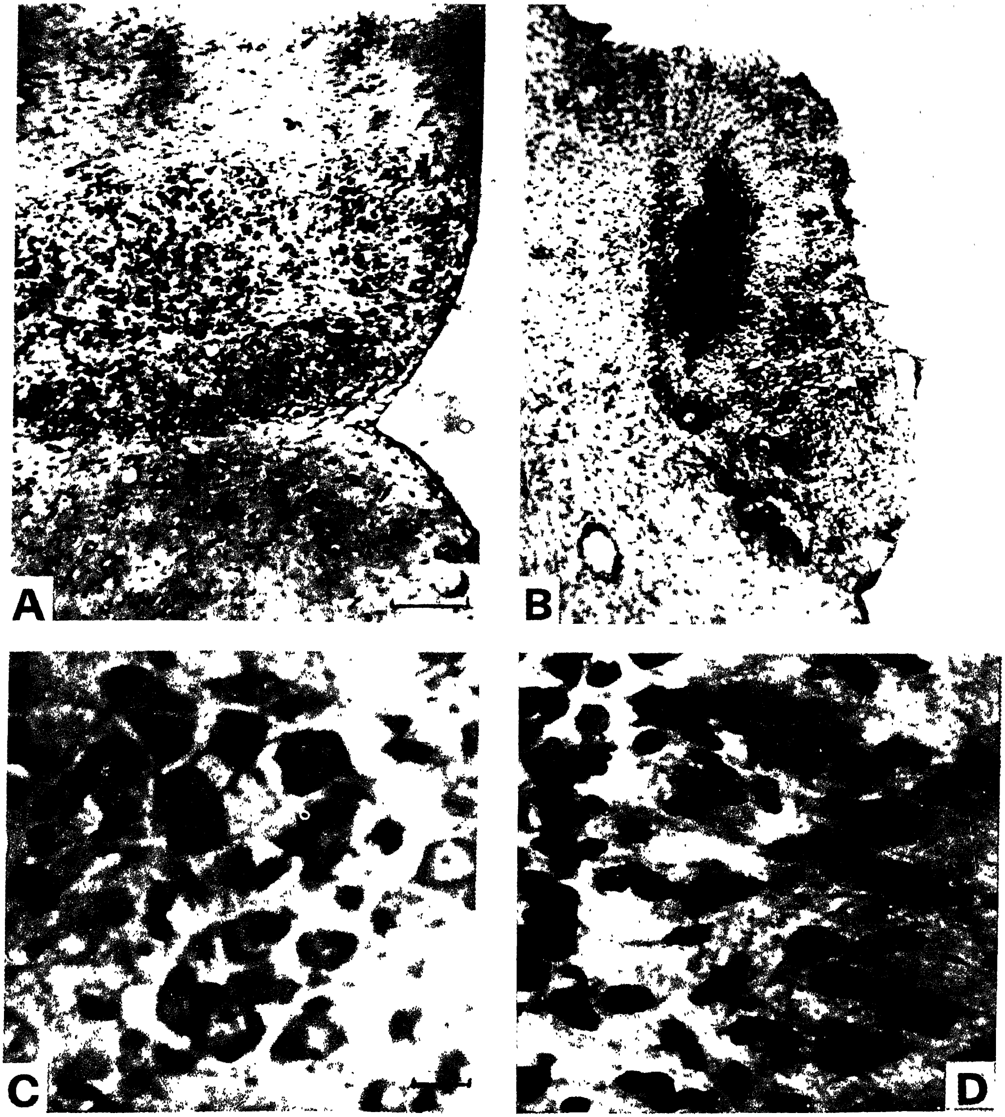


Figure 5 Rat hippocampal grafts developing for 8 weeks in the rabbit septum. Nissl's staining. **A,** The graft in the control animal not subjected to electrophysiological investigation; **B,** The graft after 7 days of electrophysiological investigation. Necrotic area and heavy lymphocyte infiltration are present. (Bars = 1 mm (above) and 40 μm (below)

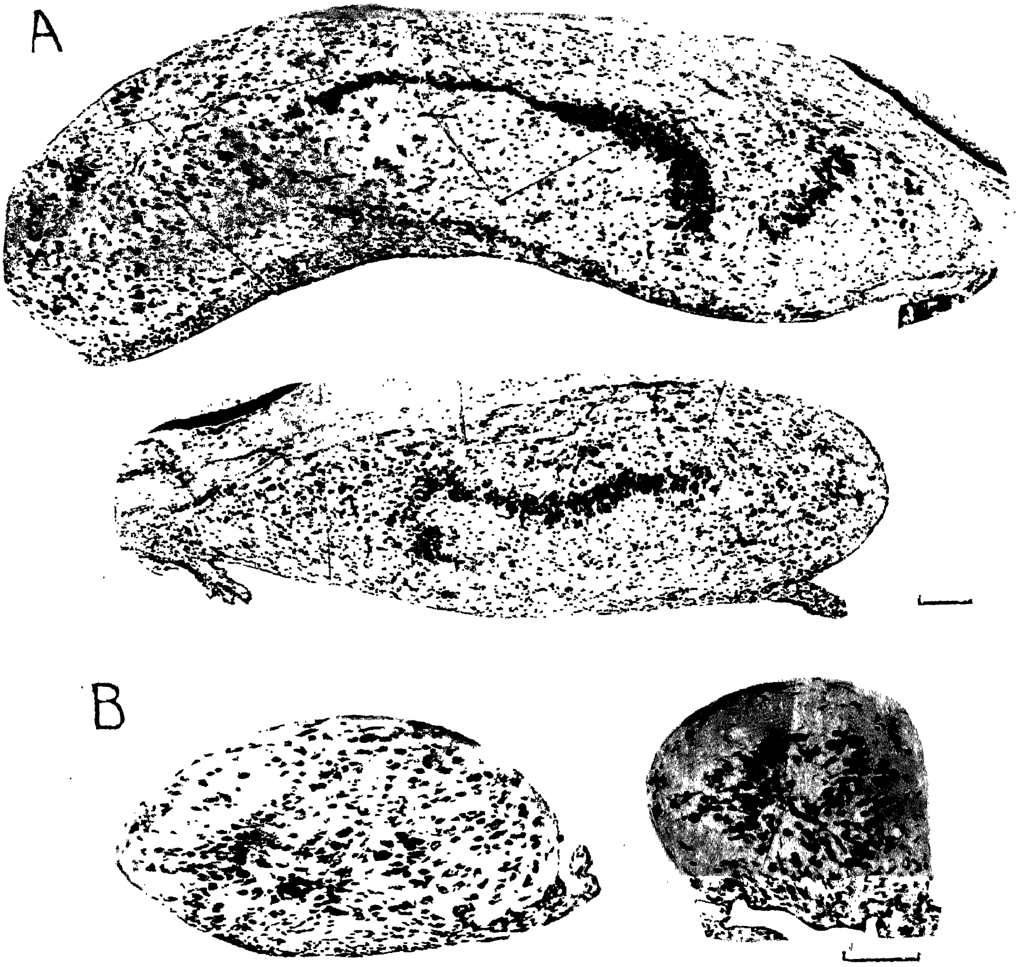


Figure 6 Typical examples of the hippocampal grafts, developing in the anterior eye chamber of the rats of different strains. **A** Wistar recipients. Well organized pyramidal layer is present. Scale-200 μm. **B** WAG recipients. Organization of the cells into layer is absent. (Scale - 100 μm). In all photographs lower part is oriented towards iris. Time after grafting-12 weeks

rounded, not elongated shape. In all WAG rats exept one, the organotypic layered organization of the hippocampal pyramidal cells was absent. Though many neurons had pyramidal characteristics their dendrites were not oriented and the cells were grouped into diffuse clusters (figure 6). There were no signs of active immune response but as all conditions of the grafting, exept the strain of the hosts were identical, it seems, that the genetic differences caused this lack of developmental expression of hippocampal architectonics, possibly by intrusion of immune influences at the early stages after the grafting.

The above data indicate once again that the immunological privilege of the brain and eye is only relative and limited. The complex factors working against the brain immunoprivilege can be listed as follows:

(i) Introduction of the graft is necessarily connected with interruption of the blood-brain barrier. Some experiments show that re-establishement of the barrier may take a long time (up to two weeks) after transplantation of the nervous tissue (Svengaard et al. 1975, Saburina et al. 1987). The barrier is not re-established after grafting of non-nervous (e.g., endocrine) tissue, which induces development of tissue-specific perforated capillaries (Zhuravleva et al. 1984, Rosenstein 1987).

(ii) While the brain cells do not express the MHC antigens, endothelial cells may express them in normal and pathological states (Baker et al. 1987, Traugott et al. 1985, Rose et al. 1987). Thus, they may act as the antigen-presenting cells for the immune system.

(iii) Activated lymphocytes, producing lymphokines, can penetrate blood-brain barrier (Wekerle et al. 1987, Fontana et al. 1987). Activation of the immune system (e.g., by peripheral grafting) leads to rejection of the intrabrain graft (Raju & Grogan 1977, Houston et al. 1987).

(iv). Under the influence of α-interferon, synthetized by activated lymphocytes penetrating into the brain parenchyma, astrocytes and oligodendrocytes express the MHC calss I and II antigens and may act as antigen-presenting cells (Wong et al. 1984, Hirsch et al. 1983, Suzumura & Silberg 1987).

(v) Development of the immune response correlates with degree of incompatibility of donor and recipient (Mason et al. 1986), but defects of brain immuno-protection might trigger even autoimmune responses. The rare cases of tissue-specific autoimmune response, provoked by allograftig of the hippocampal tissue, which has high immunogenicity (Felten & Felten 1987), were observed by Bragin (unpublished observations).

All these facts call for necessary caution, especially with clinical use of transplantation of endocrine and fetal nervous tissue, but should not lead to a pessimistic view of the perspectives of neurografting.

Some suggestions for overcoming the limitations of immunological privilege of the brain are as follows:

(i) Of course, immunosuppression, at least for the period necessary for re-establishement of the blood-brain barrier, might be used.

(ii) Brain cell suspensions prepared with the help of cytophotometric cell sorting can be used for the grafting. This may help to eliminate endothelial and ependymal elements as probable antigen-presenting cells.

(iii) Preliminary cultivation of the tissue *in vitro* is widly used in peripheral grafting as it decreases immunogenicity of the tissue. Cultivation of pieces of the nervous tissue serve the same purposes, persumably decreasing the number of endothelial cells in the nervous tissue culture. It is possible that similar effects explain better survial of the nigral grafts after their preliminary prolonged cooling, described by Tandon (this issue)

(iv) Mixed suspension of endocrine and nervous cells can be suggested as a possible way for induction of barrier capillaries in cases of transplantation of endocrine tissue.

(v) Autotransplantation of genetically engineered recipients's cells (e.g., fibroblasts) in which the genes responsible) for production of the necessary neuroactive substances are introduced may hold future promise (Selden et al. 1987, Gage et al. 1987).

Types of Compensatory Influences of the Intrabrain Grafts

One of the problems of neurografting, which is still not quite clear, is the mechanism of functional compensation by the graft and degree of its specificity. The pioneering work of Björklund and his collegues showed that behavioural defects induced by 6-OHDA lesions of substantia nigra can be abolished by grafting the nigral tissue to the caudate nucleus (Björklund et al. 1980). This correlated to survial of the grafted dopaminergic neurons (Schultzberg et al. 1984) and biochemical detection of increased levels of dopamine (Schmidt et al. 1982). These facts were enthusiastically accepted as demonstration of possibility of specific functional compensation by grafting the appropriate nervous tissue. Recent clinical application of the intrabrain grafting in incurable Parkinson patients is based upon this belief.

It must be noted, that during these series of experiments no tissue control was used. Recently, some facts have accumulated which raise some questions regarding the nature of compensation following neurografting.

(i) Sometimes the observed behavioural compensation is very rapid (one week after the surgery), which is definitely not enough for establishement of functional connections (Ermakova 1987).

(ii) In some cases of obvious behavioural compensation no graft could be found on histological examination (Stein et al. 1987, Terasawa et al 1987). Necrosig of the grafted adrenal medulla was observed in *post-mortem* investigation in two Parkinson patients, who showed improvement of their motor function after the grafting.

(iii) Some authors do not find any connections of the grafted dopaminergic neurons with the host caudate nucleus (Walker & McAlister 1987, Bing et al. 1988). though the compensation is present.

(iv) Compensation was observed after transplantation of non-specific, and even of non-nervous tissue (Kesslak et al. 1986, Pezzoli et al. 1988).

How can all these facts be explained? Possibly, they may be regarded primarily as a result of influence of the neurotrophic factors, produced by the lesioned brain (Manthorpe at al. 1983, Needels et al. 1986). In cases, in which embryonal tissue is introduced into the lesioned brain, recovery may also be helped by similar and additional active trophic substances synthetized by the graft (Haun & Cunningham 1984, Sofroniew et al. 1986, Polezhaev et al. 1986). This may serve as a trigger for mobilization of reserve functions of the lesioned brain (induction of the growth-associated proteins, stimulation of growth cones proliferation, activation of metabolic processes).

Nevertheless, the problem of specificity in compensation of the brain functions remains. Only recently the work, demonstrating specificity of compensation by grafts appeared: it showed that homolateral circling movements, evoked by metamphetamine in rats with unilateral destruction of substantia nigra are abolished by nigral, but not by raphe or striatal grafts (Dunnet et al. 1988).

Similar research, using another behavioural model, was performed earlier in our Department. Investigations by Gromova and Semyonova showed that perinatal neurotoxic lesion of the locus coeruleus noradrenergic (NA) neurons by 6-OHDA in rats resulted in stable abnormalities of behaviour. The NA-deprived rats in adult state have strong reduction of exploratory activity, measured in the open field as number of the crossed squares (horizontal activity) and number of rearings (vertical activity). In addition these animals have so-called "sensory inattention"-decreased orienting response to tactile stimulation of the body surface and to moving visual stimuli. Besides, as NA-system of the brain has primary importance for the learning based upon negative emotions, the rats have abnormal paradoxical behaviour in situation of frustration: while the normal rats, trained to run to obtain food reinforcement, strongly decrease the speed of running after sudden 10-fold decrease in amount of reinforcement, the NA-deprived rats even increase the speed of running (Gromova 1980, Gromova et al. 1985).

The NA-deprived adult rats received the grafts of embryonal (E17-18) tissue of locus coeruleus bilaterally into the frontal cortex. Three control groups of rats were used: intact normal; 6-OHDA-treated sham-operated (bilateral injection of saline into the frontal cortex); 6-OHDA-treated with bilateral grafts of the embryonal

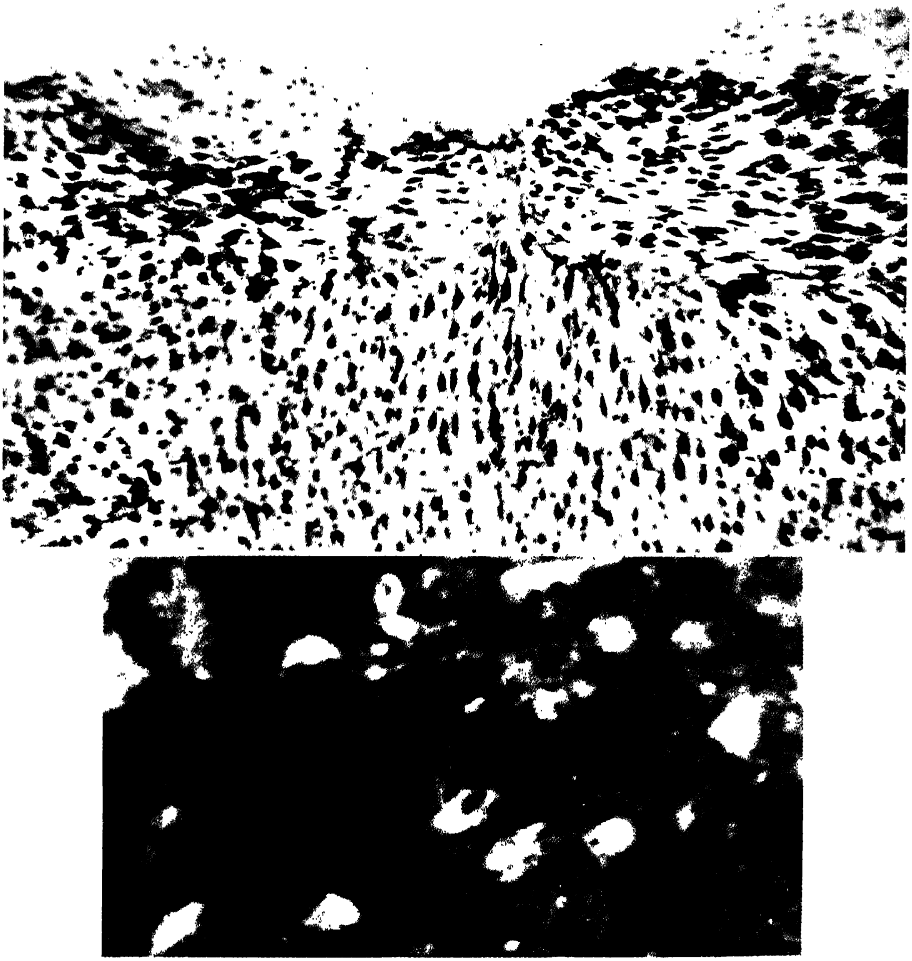


Figure 7 Locus coeruleus graft in the neocortex (Nissl staining); the track of the injecting needle is seen in the center. Horiontally-orieted spindle-shaped cells, typical for dorsolateral part of the locus coeruleus spread on both sides of the track above vertically-oriented neocortical pyramidal cells. The glial accumulation at the interface is absent (x 40). Below the fluorescent neuronal somata whithin the intracortical graft of locus coeruleus (Falck's method) (x 200)

hippocampal tissue, which does not contain NA neurons. Behavioural testing was performed beginning at one month after the grafting and up to 9 months postoperative. After sacrifice the frontal part of the forebrain was used for histological investigation (Cresyl Violet or Falck's method). The caudal part of the neocortex and brain stem were used for biochemical microfluorimetric measurement of NA concentration. All investigations were performed by four independent groups of investigators (transplantation—A G Bragin, behavioural studies—T P Semyonova and N I Grischenko, histology—I V Nesterova, and biochemistry—T M Tretyak, A V Kulikov and G N Smirnova) by double-blind method.

In all grafted animals, the NA-containing cells of the locus coeruleus were present in the frontal neocortex (figure 7). Biochemical testing showed that the NA concentration in the neocortex of the locus coeruleus-grafted animals was 272.45 ng/mg of wet tissue (range—180-440 ng/mg), which is much closer to the normal concentration (450.19 ng/mg) and significantly differes from both 6-OHDA-treated control groups (70.40 ng/mg for sham-operated group and 40.23 ng/mg for rats with hippocampal grafts, ($P<0.01$) (figure 8A). Corresponding stable normalisation of behaviour was observed in locus coeruleus-grafted rats. Their exploratory activity in the open field did not differ from that of intact animals, but was significantly higher than in both 6-OHDA-treated group of control ($P<0.001$ for horizontal activity; $P<0.05$ for vertical activity) (figure 8 B,C). Their sensory attention to tactile and visual stimuli also improved markedly (figure 8E). Orienting reaction was nearly normal, but slight improvement of attention to tactile stimuli was observed also in the rats with hippocampal grafts. Frustration test showed that while normal rats run more slowly to the decreased amount of food reingorcement, the 6-OHDA-treated animals paradoxically increased the speed of running. The same effect is present in animals with hippocampal grafts, but expression of frustration in animals with locus coeruleus grafts is indistinguishable from that of intact group (figure 8F). Thus, in this case several tests convincingly show nor-

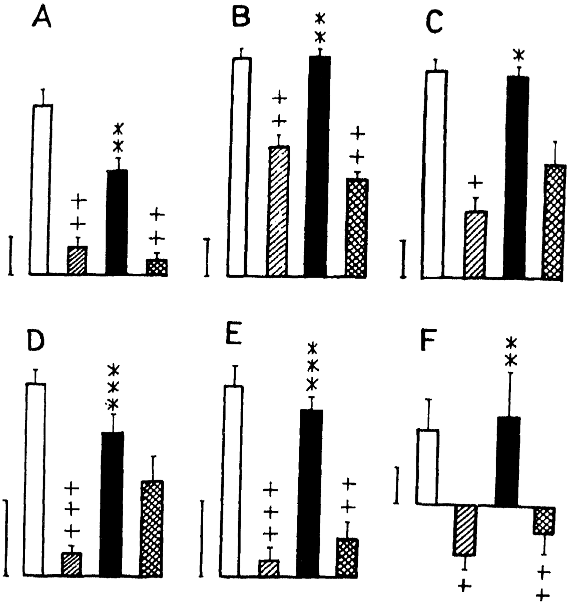


Figure 8 Biochemical and behavioural effects of the locus coeruleus grafting into the neocortex of 6-OHDA-treated rats. Empty columns-intact animals; hatched columns-sham-operated animals, perinatally treated with 6-OHDA; black columns 6-OHDA-treated animals with the locus coeruleus grafts; double hatched columns-6-OHDA-treated animals with hippocampla grafts. **A,** Neocortical concentration of noradrenaline. Scale 100 ng/mg of wet tissue; **B,** Horizontal exploratory activity (number of the crossed squares in the opened field per 3 min time of observation). Scale-20 crossings; **C,** Vertical explorator activity (number of rearings per 3 min). (Scale 1 rearing). **D,** Intensity of orienting reaction to tactile stimulation of the body surface (Scale 1 conventional unit); **E,** The same for moving visual stimulus; **F** Coefficient of frustration t_2-t_1/t_1, where t_1-time of running to large food reinforcement; t_2-time of running after tenfold decrease in amount of reinforcement. Significance of differences relative to intact contyrol is shown for 6-OHDA treated sham-oprated and hippocampally-grafted groups: + + + $P<0.001$; + + $P<0.5$. Significance of differences relative to sham-operated group: $P<p.001$; $P<0.01$; $P<0.5$.

malization of specific behavioural defects of 6-OHDA lesioned animals only by the locus coeruleus grafts, though slight tendency to normalization can be detected in some tests in animals with hippocampal grafts (figure 7 C and D). Evidence of the tissue-specific effect of the graft was obtained recently in behavioural investigation by Gromova, Semyonova and Grischenko in animals with 5,7-DOT lesions of serotoninergic system and grafts of embryonal raphe tissue compared to the animals with the hippocampal grafts.

Summarizing general data on the functional effects of neurografting it is necessary to indicate that the evidence of adverse influence of the grafts (e.g. prevention of spontaneous regeneration and functional compensation by the glial scar) are very limited. On the contrary, the reports on positive effects of neurografting are multiple. These positive effects can be summarized as follows·

(i) The grafts may serve as tissue bridges promoting the growth and reinnervation of the normal targets by the lesioned neurons which otherwise would die in the course of retrograde degeneration (Kromer et al. 1981).

(ii) The grafts (irrespective of their structural origin and place of transplantation) may exert general non-specific neurotrophic influence even in the cases of diffuse pathology of the brain (e.g. anoxia; see Alexandrova.

(iii) Grafts of the biochemically specialized neuronal systems synthetizing neuromodulators (biogenic amines, acetylcholine) or neurohormons (e.g., vasopressine) can participate in restoration of the corresponding systemic functions. Probably the accurate restoration of architectonics of the lesioned connections and establishment of synapses are not always necessary in such cases, as extrasynaptic route may be used (e.g. Becker & Freed 1988).

(iv) The possibility of restoration of functions of such areas of the brain, where specific structural organization is supposed to be of critical importance (neocortex, cerebellum, hippocampus) is still not clear, though some positive results are reported (Chang et al. 1986, Levin et al. 1987 Yirimiya et al. 1988, (see also Bragin, p).

The clear understanding of certain limitations of the neurografting together with detailed experimental investigation of the possibilities listed above may open new (and possibly yet unsuspected vistas in the application of this method.

References

Armant R and Turner J E 1988 Cross-species grafting of embryonic mouse and grafting of older postnatal rat retinas into the lesioned adult rat eye: the importance of cyclosporin A for survival; *Devel. Brain Res.* **41** 303-307

Baker B J, Puklavce M and Charlton H M 1987 The vascularization of intracerebroventricular grafts visualised by monoclonal antibodie. *The 2nd Int. Symp. Neurotransplantation*, Rochester

Bauer H C and Hurenbach U 1986 Murine cortical brain cells are autoantigenic from a distinct developmental stage onwards; *J. Neuroimmunol.* **12** 1-13

Becker J B and Freed W J 1988 Adrenal medulla grafts enhance functional activity of the striatal dopamine system following substantia nigra lesions; *Brain Res.* **462** 401-406

Bickford-Wimer P, Granholm A C, Bygdeman M, Hoffer B, Olson L, Seiger A and Strömberg I 1987 Human fetal cerebellar and cortical tissue transplanted to the AEC of athymic rats: electrophysiological and structural studies; *Proc, Nat. Acad. Sci. USA* **84** 5957-5961

Bing G, Netter M F D, Hansen J T and Gash D M 1988 Comparison of adrenal medullary, carotid body and PC 12 cell grafts in 60 HDA lesioned rats; *Brain Res. Bull.* **20** 399-406

Björklund A, Stenevi U 1979 Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants; *Brain Res.* **177** 555-560

——, Dunnett S B, Stenevi U, Lewis M E and Iversen S D 1980 Reinnervation of the denervated striatum by substantia nigra transplants: functional consequences as revealed by pharmacological and sensorimotor testing; *Brain Res.* **199** 307-333

Bolam J P, Freund T F, Björklund A, Dunnett S P and Smith A D 1987 Synaptic input and local output of dopaminergic neurons in grafts that functionally reinnervate the host neostriatum; *Exp. Brain Res.* **68** 131-146

Chang F L F, Steedman J G and Lund R D 1986 The lamination and connectivity of embrynic cerebral cortex transplanted into newborn rat cortex; *J. Comp. Neurol.* **244** 401-411

Daniloff J K, Low W C, Bodony K P and Wells J 1985 Cross-species neural transplants of embryonic septal nuclei to the hippocampal formation of adult rats; *Exp. Brain Res.* **59** 73-82

Das G D, Altman J 1972 Studies on the transplantation of developing neural tissue in the mammalian brain. I Transplantation of cerebellar slabs into the cerebellum of neonatal rats; *Brain Res.* **38** 233-249

Dunnett S B, Hernandez T D, Summerfield A, Jones G H, Arbuthnott G 1988 Graft-derived recovery from 6-OHDA lesions specificity of ventral mesencephalic graft tissue; *Exp. Brain Res.* 71 411-424

Ermakova I V 1981. Possibility of restoration of the defective food search as a result of neurografting; *Zhurn. Vyss. Nervn. Deyat.* **37** 168-171 (Russian)

Felten D L, Felten S Y 1987 Immune interactions with specific neural structures; *Brain Behav. Immunity* 1 279-283

Fontana A, Frei K, Bodmer S and Hoffer E 1967. Immune-mediated encephalitis: On the role of antigen-presenting cells in brain tissue; *Immun. Rev.* **100** 185-201

Foster T C, Christian E P, Hampson R E, Campbell K A and Deadwyler S A 1987. Sequential dependencies regulate sensory evoked responses of single units in the rat hippocampus; *Brain Res.* **408** 86-96

Fox S E and Ranck J B 1981 Electrophysiological characteristics of hippocampal complex-spike cells and theta cells; *Exp Brain Res.* **41** 339-410

Frohman E M, Vayuvegula B, van der Noort S, Gupta S 1988 Norepinephrine inhibits gamma-interferon-induced MHC class II (Ia) antigen expression on cultured brain astrocytes; *J. Neuroimmunol.* **17** 89-101

Gage F H, Wolff J A, Rosenberg M B, Xu L, Jee J K, Shults C and Friedmann T 1987 Grafting genetically modified cells to the brain: possibilities for the future; *Neurosci.* **23** 795-807

Gromova E A 1980 Emotional Memory and its Mechanisms; [Moscow. Nauka (Russian)]

——, Semyonova T P and Grischenko N I 1985 Effects of acute and chronic lesion of chatecholaminergic systems by 6-OHDA on behaviour of rats; *Zhurn. Vyss. Nervn. Deyat.* **35** 1133-1139 (Russian)

Hart D A and Fabre J W 1979 Quantitative studies on the tissue distribution of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain; *J. Exp. Med.* **153** 347-361

Haun F and Cunningham T J 1984 Cortical transplants reveal CNS trophic-interactions in situ; *Dev. Brain Res.* **15** 290-294

Head J R, Billingham R E 1985 Immunologically privileged sites in transplantation immunology and oncology; *Persp. Biol. Med.* **29** 115-131

Hickey W F and Kimura H 1987 Graft-vs.-host disease elicits expression of class I and class II histocompatibility antigens and the presence of scattered T-lymphocytes in rat CNS; *Proc. Natl. Acad. Sci. USA* **84** 2082-2086

Hirsch M R, Wietzerbin J, Pierres M, Goridis C 1983 Expression of Ia antigens by cultured astrocytes treated with gamma-interferon; *Neurosci. Lett.* **41** 199-204

Houston M B, Kunz H W, Gill T J and Lund R D 1987 Neural xenograft rejection in the CNS; *J. Neuroimmunol.* **16** 79

Inoue H, Kohsaka S, Yoshida K, Ohtani M, Toya S and Tsukada Y 1985 Cyclosporin A enhances the survivability of mouse cerebral cortex grafted into the third ventricle of rat brain; Neurosci. Lett. **54** 85-90

Kamo H, Kim S U, McGeer P L and Shi D H 1986 Functional recovery in a rat model of Parkinson's disease following transplantation of cultured human sympathetic neurons; *Brain Res.* **397** 372-376

Kaplan H J and Streilein J W 1977 Analysis of immunologic privilege within the anterior chamber of the eye; *Transplant. Proceed.* 9 1193-1195

Kesslak J P, Nieto-Sampedro M, Globus J, Cotman C W 1986 Transplants of purified astrocytes promote behavioral recovery after frontal cortex ablation; *Exp. Neurol.* **92** 377-390

Kromer L F, Björklund A and Stenevi U 1981 Regeneration of the septohippocampal pathways in adult rats is promoted by utilizing embryonic hippocampal implants as bridges; *Brain Res.* **210** 173-200

Levin B E, Dunn-Meynell A and Sved A F 1987 Functional integration of fetal cortical grafts into the afferent pathway of the rat somatosensory cortex (Sm I); *Brain Res. Bull.* **19** 723-734

Lisak R P, Hiragama M, Kuchmy D, Rosenzweig A, Kim S U, Plasure D E and Silberberg D H 1983 Cultured human and rat oligodendrocytes and rat Schwann cells do not have immune response gene associated antigen (Ia) on their surface; *Brain Res.* **289** 285-292

Manthorpe M, Nieto-Sampedro M, Skaper S D, Lewis E R, Barbin G, Longo F M, Cotman C W and Varon S 1983 Neuronotrophic activity in brain wounds of the developing rat. Correlation with implant survival in the wound cavity; *Brain Res.* **267** 47-56

Mason D W, Charlton H M, Jones A J, Lavy C B D, Puklavec M, Simmonds S J 1986 The fate of allogenic and xenogenic neuronal tissue transplanted into the third ventricle of rodents; *Neuroscience* **19** 685-694

Masuko S and Shimada Y 1983 Neuronal cell-surface specific antigen(s) is expressed during the terminal mitosis of cells destined to become neuroblasts; *Devel. Biol.* **96** 396-404

Medawar P B 1948 Immunity to homologous grafted skin. III. The fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye; *Brit. J. Exp. Pathol.* **29** 58-69

Needels D L, Nieto-Sampedro M and Cotman C W 1986 Induction of a neurite-promoting factor in rat brain following injury or deafferentation; *Neuroscience* **18** 517-526

Olson L and Seiger A 1972 Brain tissue transplanted to the anterior chamber of the eye. I. Fluorescence histochemistry of immature catecholamine and 5-hydroxytryptamine neurons reinnervating the rat iris; *Zellforschung.* **135** 175-194

Pezzoli G, Fahn S, Dwork A, Truong D D, de Yebenas J, Jackson Lewis V, Herbert J and Cadet J L 1988 Non-chrom-tissue plus NGF reduces experimental parkinsonism in aged.rats; *Brain Res.* **459** 398-403

Polezhaev L V, Alexandrova M A and Girman S V 1986 Normalization of dystrophic brain cortex neurons after hypoxia and transplantation of embryonic nervous tissue in rats; *J. Hirnforschung.* **27** 501-514

Raju S and Grogan J B 1977 Immunologic study of the brain as a privileged site; *Transplant. Proc.* **9** 1187-1191

Rao K, Kunz H W, Gill T J and Lund R D 1987 MHC-dependent neural allograft rejection; *J. Neuroimmunol.* **16** 143

Rawlins J N P, Feldon J and Gray J A 1979 Septo-hippocampal connections and the hippocampal theta-rhythm; *Exp. Brain Res.* **37** 49-63

Rose L M, Peterson R, Mehra R and Alvord E C 1987 Endothelial Ia Expression increases prior to inflammatory cell infiltration in EAE induced in longtailed macaques; *J. Neuroimmunol.* **16** 148

Rosenstein J M 1987 Adrenal medulla grafts produce blood-brain barrier dysfunction; *Brain Res.* **414** 192-196

Saburina I N, Konorova I L, Gannushkina I V and Polezhaev L V 1987 Permeability of the blood-brain barrier to blood globulines after allotransplantation of the nervous tissue into the brain of adult normal and hypoxic rats; *Docklady Academii Nauk* **292** 209-211

Selden R F, Skoskiewiez M J, Howie K B, Russell P S and Goodman H M 1987. Implantation of genetically engineered fibroblasts into mice: implications for gene therapy; *Science* **236** 714-716

Schmidt R H, Ingvar M, Lindvall O, Stenevi U and Björklund A 1982. Functional activity of substantia nigra grafts reinnervating the striatum: neurotransmitter metabolism and [14/c] 2-deoxy-D-glucose autoradiography; *J. Neurochem.* **38** 737-748

Schuller-Petrovic S, Gebhart W, Lassman H, Rumpold H and Kraft D 1983 A shared antigenic determinant between natural killer cells and nervous tissue; *Nature* **306** 179-181

Schultzberg M, Dunnett S B, Björklund A, Stenevi U, Hokfelt T, Dockray G I and Coldsein M 1984 Dopamine and cholecystokinin-immunoreactive neurons in measencephalic grafts reinnervating the neostriatum: evidence for selective growth regulation; *Neurosciences* **12** 17-32

Sofroniew M V, Isacson O and Björklund A 1986 Cortical grafts prevent atrophy of cholinergic basal nucleus neurons induced by excitotoxic cortical damage; *Brain Res.* **378** 409-415

Suzumura A and Silberg D H 1987. MHC antigen expression on glial cells; *J. Neuroimmunol.* **16** 168

Stein D G, Pallatucci C, Slavin M, Labbe R and Curran E 1987 Neurotrophic effects of fetal brain tissue transplants on recovery of cognitive performance in rats with frontal and motor cortex lesions; "Functional Aspects of Neuronal Transplantation in the Mammalian CNS"; *Absts IBRO Satellite Symposium,* Pecs

Steward P A and Wiley M J 1981. Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: A study using quail-chick transplantation chimeras; *Develop. Biol.* **84** 183-192

Svengaard N A, Björklund A, Hardebo J E and Stenevi U 1975 Axonal degeneration associated with a defective blood-brain barrier in cerebral implants; *Nature* **255** 334-337

Terasawa E, Uno H, Krook C ,Brigham P A and Gash D A 1987 Fetal neural transplantation improved spastic motor paralysis with hemicortical lesions in a rhesus monkey; *Absts 2nd Int. Symp. Neural Transplantation,* Rochester

Thompson W G 1890 Successful brain grafting *New York Medical J* 701-702

Traugott U, Scheinberg L C and Raine C S 1985 On the presence of Ia-positive endothelial cells and astrocytes in multiple sclerosis lesions and its relevance to antigen presentation; *J. Neuroimmunol.* **8** 1-14

Vinogradova O S 1975 The role of the limbic system in registration of information: Facts and hypotheses; in *The Hippocampus ed. K Pribram R and Isaacson* (New York: Pergamon Press) Vol. II 3-70

Vinogradova O S, Brazhnik E S, Karanov A M and Zhadina S D 1980 Analysis of neuronal activity in rabbit's septum with various conditions of deafferentation; *Brain Res.* **187** 354-368

Walker P D and McAllister J P 1987 Minimal connectivity between neostriatal transplants and the host brain: *Brain Res.* **425** 34-44

Walker R F and Codd E E 1985. Neuroimmunomodulatory interactions of norepinephrine and serotonin; *J. Neuroimmunol.* **10** 41-58

Wekerle H, Sun D, Oropeza-Wekerle R L and Meyermann R 1987 Immune reactivity in the nervous system: modulation of T-lymphocyte activation by glial cells: *J. Expl. Biol.* **132** 43-57

Wong G H W, Bartlett P F, Clark-Lewis I, Battye F and Scharder J W 1984 Inducible expression of H-2 and Ia antigens on brain cells; *Nature* **310** 688-691

Yirmiya R, Zhou F C, Holder M D, Deems D A and Garcia J 1988 Partial recovery of gustatory function after neural tissue transplantation to the lesioned gustatory neocortex; *Brain Res. Bull.* **20** 619-625

Young I D E and Liu C C 1988 How do cytotoxic T-lymphocytes avoid self-lysis; *Immunol. Today* **9** 14-15

Zhuravleva Z N, Bragin A G and Vinogradova O S 1984. Organization of the nervous tissue (hippocampus and septum) developing in the anterior eye chamber: I General characteristics and non-neural elements; *J. Hirnforschung.* **25** 313-330

Zimmer J, Finsen B, Sorensen T and Poulsen P H 1988 Zenografts of mouse hippocampal tissue. Exchange of laminar and neuropeptide specific nerve connections with the host rat brain; *Brain Res. Bull.* **20** 369-379

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 125-140 (1990)

Ultracytochemical Study of the Surface Membrane Components of Nerve Cells Developing in Monolayer Cultures

G G SKIBO and L M KOVAL
Bogomoletz Institute of Physiology, Kiev (USSR)

The use of colloidal gold conjugated lectins allowed us to reveal the nature and topography of carbohydrate determinants on the nerve and glial cell surface membrane at early stages of differentiation of spinal cord and spinal ganglia cultivated cells. The differences were found in the distribution of carbohydrate determinants on the membrane of functionally different neurons and glial cells as also in the pattern of their distribution in the different parts of the neuron (soma, axon, dendrites, growth cone, contacts). Each type of neurons and each part of neurons or glia has its own "signature" on the cell surface in the form of different quantity of specific carbohydrate residue which is controlled by cell genome and the mechanism of cell synthesis.

The study of carbohydrate determinants in neurons developing in monolayer of completely isolated cells of embryonic tissue but not in infact tissue suggests that such set of carbohydrate determinants at difinite stage of differentiation of functionally different neurons reflects genetically determined processes of maturation of cytoplasmic membranes which manifest itselves in the specificity of their molecular organization.

Key Words: Ultracytochemistry, Carbohydrate, Determinants Surface membrane, Lectins Glial Cell, Never cell

Introduction

Recently information has emerged that during nerve cell development the set of glycoconjugates may vary in its different parts; the most visible changes in surface carbohydrate determinants were observed on the membrane of growth cones-the labile structures where modification of carbohydrate residues of glycoconjugates is most probable (Grunberg & Pfenninger 1986, McLaughlin & Wood 1977, Pfenninger & Maylie-Pfenninger 1981, Readler et al. 1981). It has also been suggested that carbohydrate codes of the plasmalemma for functionally different neurons might be different (Foucaud et at. 1982, Pfenninger & Maylie-Pfenninger 1979).

Thus, a study of the structure and distribution of carbohydrate determinants at different stages of the nerve cell differentiation is of considerable interest for the understanding of cellular mechanisms of neuro-ontogenesis.

This communication describes an electron-cytochemical study of the nature and topography of membrane surface glycoconjugates of the nerve cells which differentiate in monolayer culture of nerve and glial cells of the spinal cord and spinal ganglia. Colloidal gold lectins complexes were used as probes for mapping the distribution of surface membrane glycoconjugates.

Monolayer culture of spinal cord and spinal ganglia dissociated cells is one of the most convenient models for this study because it has a good contact with the liquid incubation medium and is easily accessible to macromolecular reagents.

Material and Methods

The spinal cord and spinal ganglia of 12-14 day old mouse embryos were used. At this stage of development neuroblasts of motor and sensory nuclei of the spinal cord are getting organized. Consecutive stages of neuronal development up to its mature forms in respect of functional, transmitter and structural evolution were observed (figure 1). All synaptic contacts revealed during cultivation can.be regarded as formed *de novo* (figure 2). The spinal cord and spinal ganglia were dissociated to cells which were then cultivated on the polylysine/collagen precoated plastic film enclosed in plastic Petri dishes for 2-4 weeks at 36°C in incubator with 5% CO_2 95% air (Skibo & Koval 1984). The growth and development of the cultures were monitered by light microscopy. For electron-cytochemical study of glycoconjugates of the cell surface of neurons developing in culture lectins were used as probes. For visualization, lectins were labelled with colloidal gold particles 16-20 nm. (Geoghean et al. 1977). Ultrathin sections of the cells monolayer containing neurons, glial cells and their processes were studied in the electron microscope after treatment with a variety of lectins (Roth 1983). The distribution of carbohydrate determinants both on the membrane of different parts of the neuron (cell body, dendrities, axons, growth cones) and on the surface of functionally different cells (neurons of spinal cord, spinal ganglia, glial cells) was compared. The following set of lectins was used.

(i) Helix pomatia agglutinin (HPL)
(ii) Lima bean agglutinin (LBA)
(iii) Wheat germ agglutinin (WGA)
(iv) Ricinus communis agglutinin (RCA)
(v) Lotus tetrogonolobus agglutinin (TPL)
(vi) Peanut agglutinin (PNA)

In order to check the specificity of lectin binding, the cells were treated in the presence of carbohydrate inhibitors (see Wu 1987).

Results and Discussion

The analysis of peculiarities of lectin binding specificity for certain carbohydrate determinants in the membrane of different cell elements of the cultivated neurons revealed some interesting features: (i) the presence of a gradient of distribution density of certain carbohydrate determinants within the membranes of the soma, axon and growth cone of neurons: (ii) a progressive increase in binding sites was observed from soma to the processes for N-acetyl-D—galactosamine (binding with HPL) and N-acetylglucosamine (binding with WGA) residues on the 7th day of cultivation (figure 3); (iii) an opposite tendency (a decrease in binding sites) was found for D-galactose (binding with RCA) carbohydrate residues. On the basis of these observations, it is suggested that each of the lectins is bound to a certain receptor group on the surface membrane and the location of such receptors for RCA differs from that for WGA and HPL. This conclusion seems to be valid when analysing the distribution of binding sites for different lectins in the membranes of soma, axons, dentrites and growth cones. Comparison of the binding pattern and the distribution density of the same carbohydrate determinants on the plasmalemma surface of the spinal cord and spinal ganglia neurons, reveals that these indices differ greatly among the neurons with different functions. So, HPL-Au complexes were rather numerous on the membrane of spinal cord neurons while only a few of them were seen on the surface of the spinal ganglia sensory neurons. The most distinct difference was in the distribution of RCA-Au complexes: their high density was observed on the membrane of sensory neurons and very low density on the surface of the spinal cord multipolar neuron (figure 4). On the contrary, WGA-Au conjugates, with affinity for N-acetyl-D-glucosamine residues and neuramine acid, were rarely seen on the membrane of spinal ganglia neurons but were abundant on the somatic membrane of multipolar neurons of the spinal cord (figure 5). The analysis of glycoconjugates distribution on the surface membrane of cultured neurons of the spinal cord and spinal ganglia reveal that the cells with different functions might have a carbohydrate characteristic of the surface membrane peculiar only to one type of cells. This conclusion supports the hypothesis of Sperry (1975) that the neurons may have chemical differences in the molecular com-

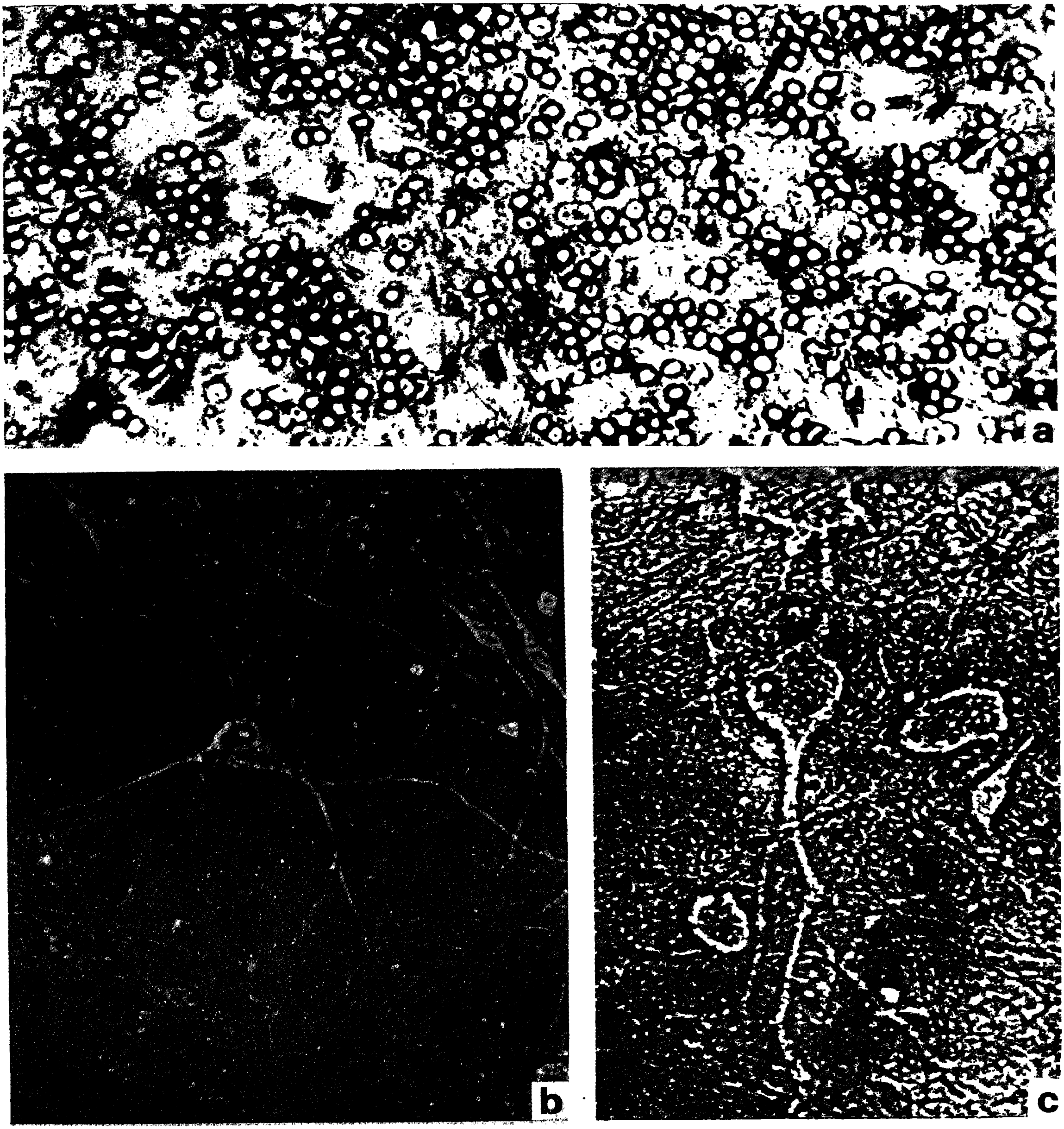


Figure 1 Living unstained preparation of cells of the spinal and spinal ganglia. **a**, a few minutes after dissociation: **b, c,** neurons after 21 days in cultivation

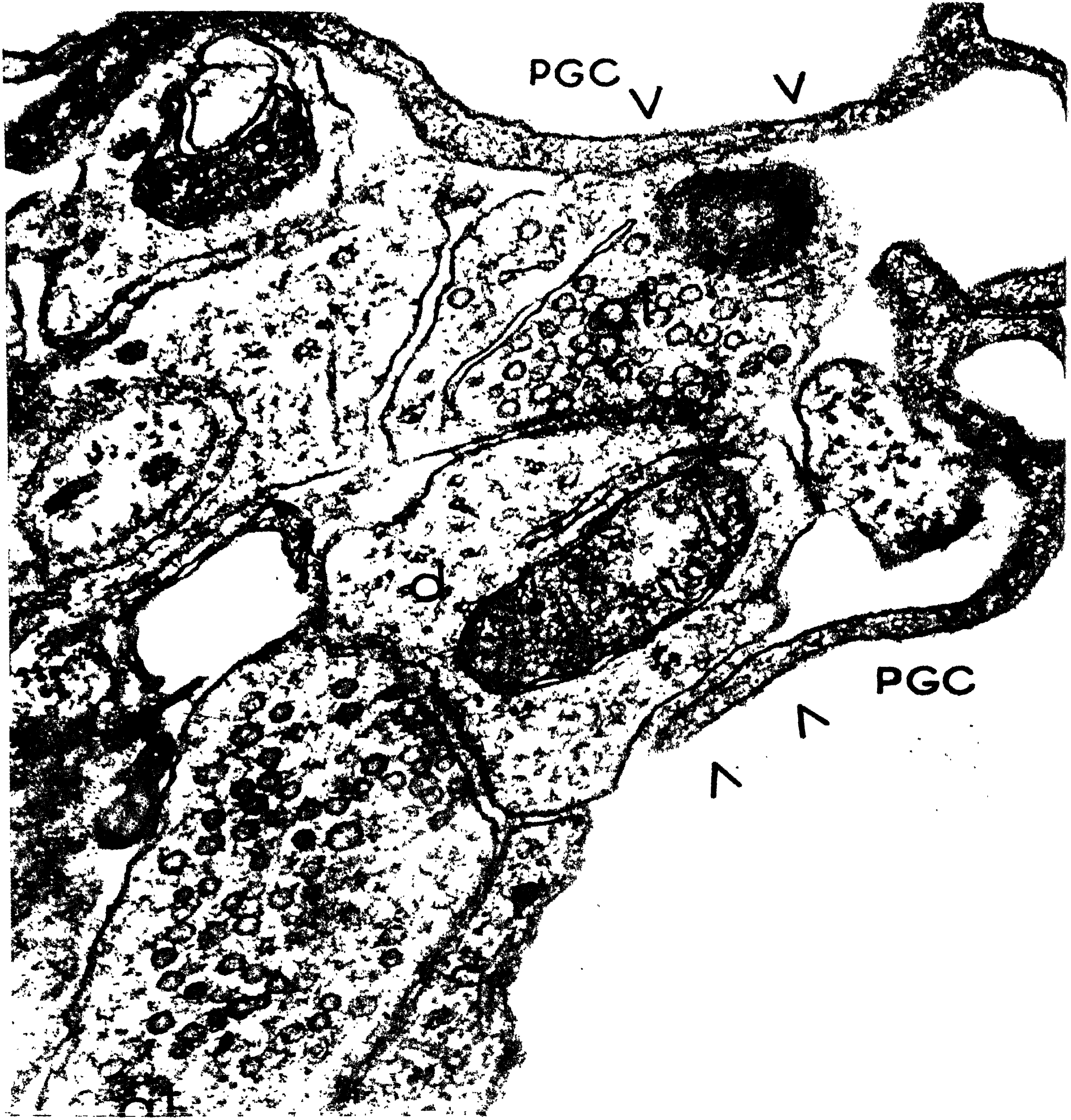


Figure 2 Synaptic complex with dendrite (d) in the center. This complex is surrouned by the glial cells protrusions (**pgc**)—shown by arrows: **at**-axon terminals.

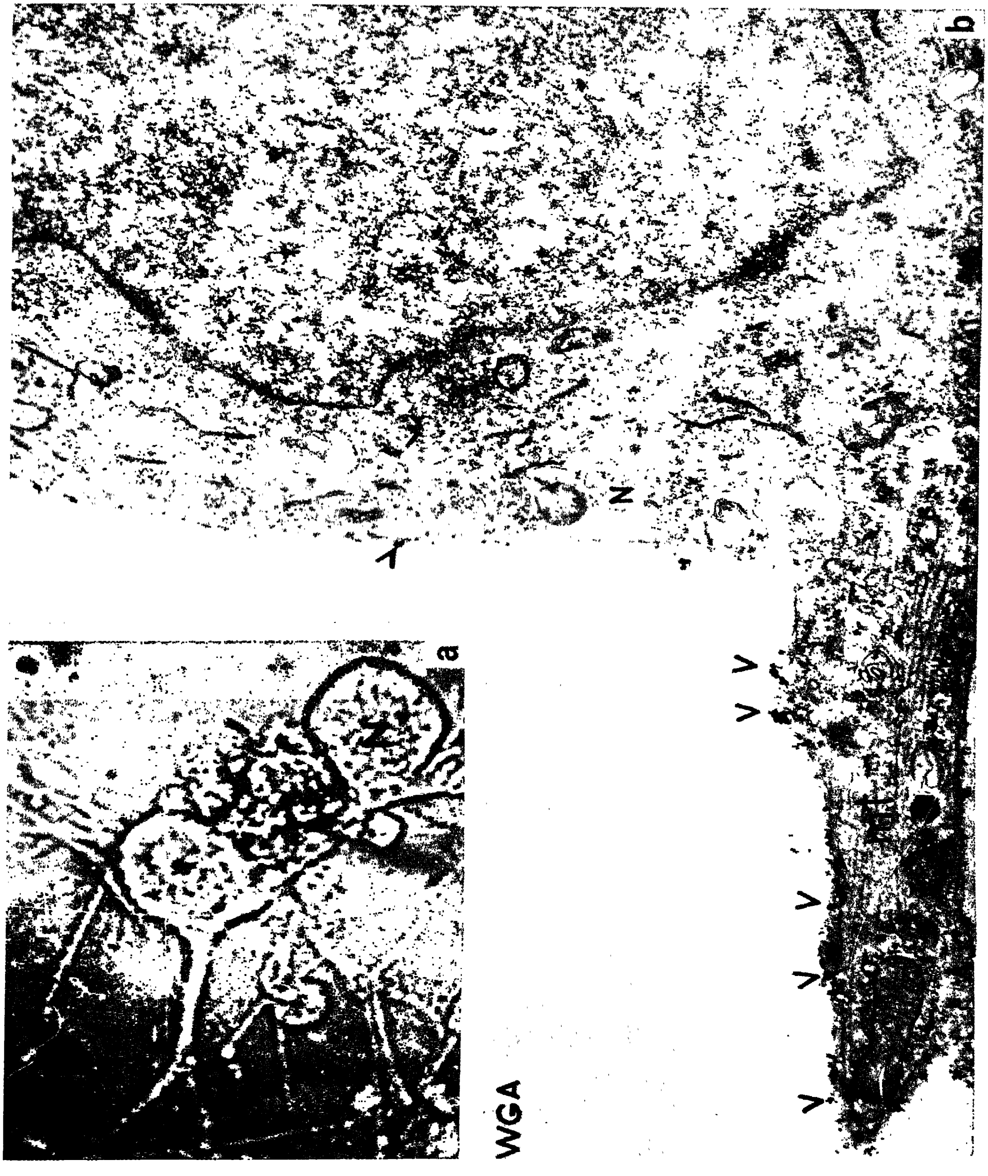


Figure 3 a-b, The distribution of WGA-Au glycoconjugates on the surface membrane of the spinal ganglia neuron. **a**, living unstained preparation; **b**, number of colloidal gold granules is greater on the neuronal outgrowth than on the neuronal soma

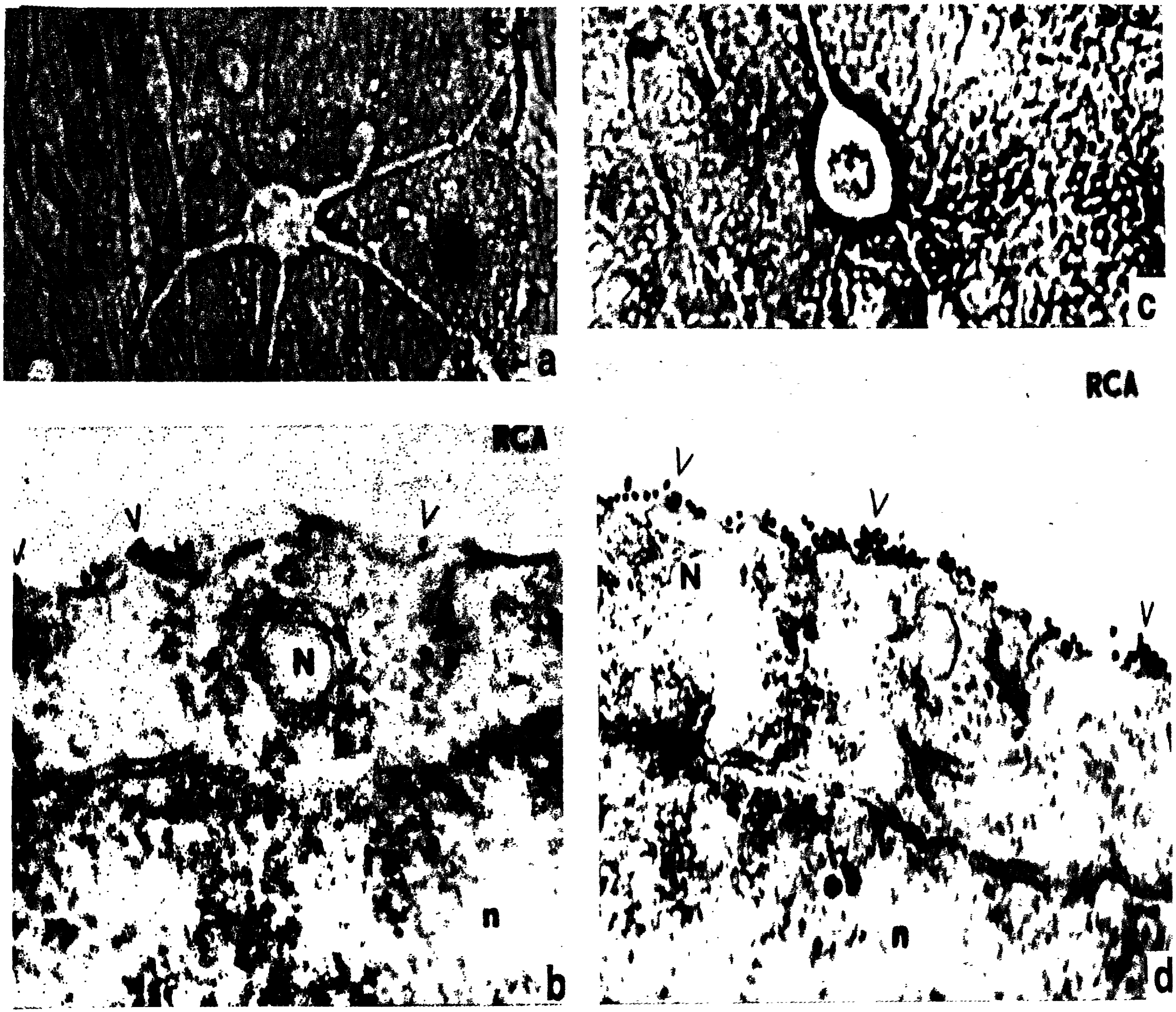


Figure 4 Different distribution of WGA-Au glycoconjugates on the surface membrane of: **a**, **b**, the spinal cord neuron; **c**, **d**, the spinal ganglia neuron **a**, **c** , living unstained preparations; 12 days *in vitro*.

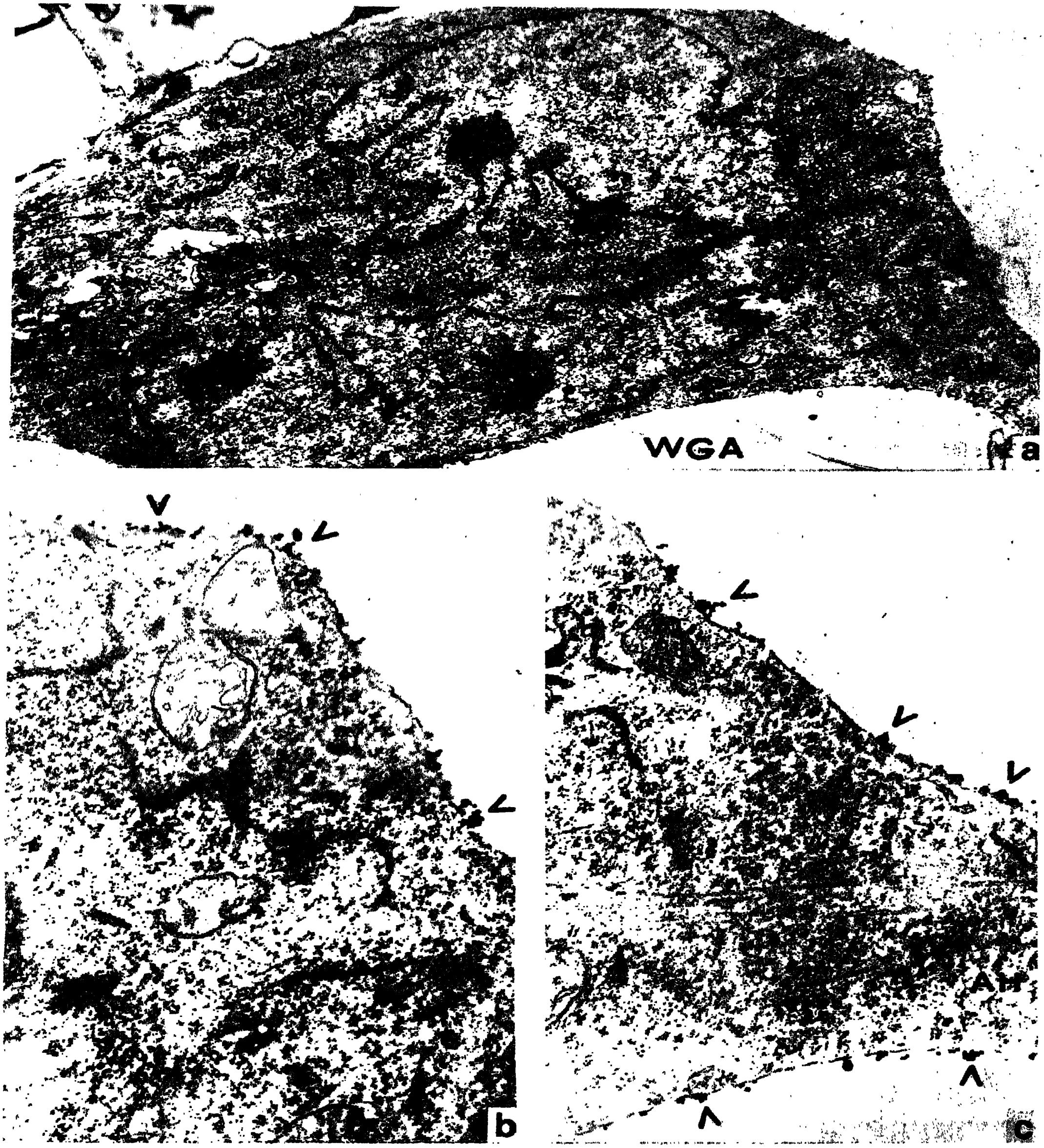


Figure 5 **a-c** The distribution of WGA-Au glycoconjugates on the surface membrane of the spinal cord neuron **a**, a spinal cord neuron and its axon hillock (**AH**); **b**, the neuronal surface membrane (the enlarged fragment of Fig. a); **c**, the axon hillock (**AH**) (the enlarged fragment of Fig. a)

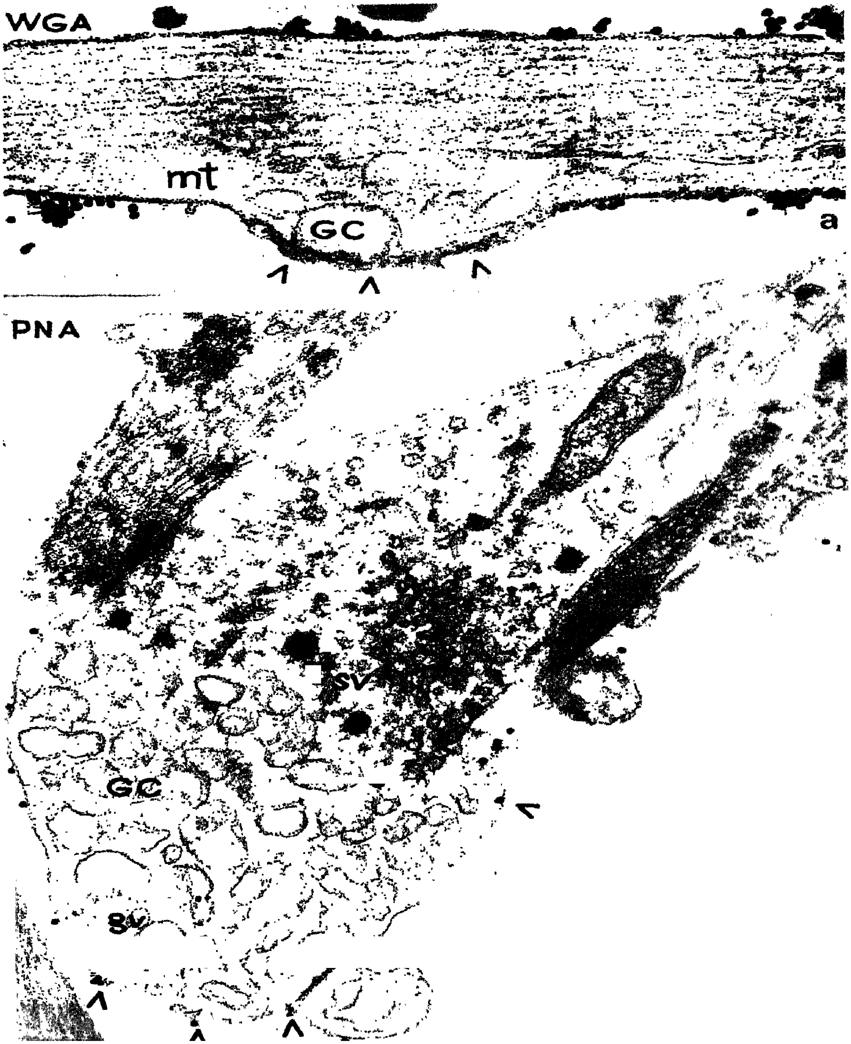


Figure 6 The distribution of WGA-Au(a) and PNA-Au(b) on the surface membrane of growth cones. Note the absence of colloidal gald granules on the surface membrane of growth cone (shown by arros): GC-Growth cone, sv-synaptic vesicles, gv-growth vesicles, mt-microtubules.

plexes on the surface which enable the axons to recognize either similar or complementary complexes on the surface of target cell. The analysis of glycoconjugates on the surface membrane of growth cones of the axons and dendrites, contrary to expectations, showed that these have either unitary binding pattern or no marker for most of the lectins used for the study except for RCA-Au binding (figures 6, 7b). It is known that the growth cone membrane has a unique property ensuring a directed growth and an accurate choice of target cell.

Comparison of the results of the present study and those obtained on pure cultures of the superior cervical ganglion (Pfenninger 1978) where binding capacity of the growth cones was the highest, would suggest that the growth cone membrane, like the somatic membrane, might possess certain specificity.

Studying the distribution of carboydrate determinants in the growth cone (especially with the RCA-Au) it was noticed that all colloidal gold granules were concentrated on the membrane of growing part of the cone identified by the presence of large growth vesicles in this region. In the proximal, forming part of the growth cone there were no such granules, thus indicating a heterogenicity of the membrane within the growth cone itself (figure 6a, b; 7a, b).

Establishment of synaptic junctions between the axon terminals and target cells is the final stage of cell differentiation and the ultimate aim of axon growth. Strict selectivity of location of synaptic contacts on certain parts of the target cells provides a specificity of neuronal connections in the nervous system. At present little is known about cytochemical changes which occur in post-and presynaptic junctional membrane during neuronal differentiation.

The use of a set of lectins labelled with colloidal gold allowed us to make interesting observations. It turned out that large amounts of Con A, LBA-Au, RCA-Au conjugates from amongst the lectins used, are observed in synaptic cleft. Since Con A has a low specificity and affinity for many carbohydrate determinants (Mannose, glucose, N-acetyl-D-glucosamine), its binding nature gives a general picture of distribution of carbohydrate determinants with monospecific lectins: RCA-Au and LBA-Au give more precise results. According to our data, despite the presence of RCA-Au conjugates (marking D-galactose) in abundance on the growing part of growth cone in the region of synaptic contacts they are located on the periphery of the synaptic cleft as if limiting it. LBA-Au conjugates (Markeing N-acetyl-D-galactose) were seen all along the extension of the synaptic cleft. Following investigation of the macromolecular architecture of the CNS synaptic contacts by means of biochemical methods, Nicolson (1976) came to the conclusion that only Con A binding determinants observed in the fraction of the CNS synaptic membranes remain in the synaptic cleft. Comparison between our results and those of Nicolson (1976) allows us to believe that together with Con A binding glycoproteins the synaptic cleft contains also N-acetyl-D-galactose residues, though in the latter case the observed difference in carbohydrate sequence may differ in the synapses of the central and autonomic nervous system. Such explanation is rather well-founded because our experimental model contained the cells of both systems.

It is known that sialation (joining of sialic or neuraminic acid) of the terminal residue of oligosaccharide chain, which serves as a signal limiting further joining of carbohydrate determinants, is a biochemical manifestation for the end of cell differentiation. Using a conjugate which can partly bind the residues of sialic acid, we did not find any granules of colloidal gold in the region of synaptic contact that may indicate immaturity of the membrane in this region. According to Rostas and Jeffery (1981), at the stage when the chick forebrain synapses seem to be morphologically mature, biochemical examination indicates their immaturity, and full maturation of postsynaptic elements takes place during the second postnatal month. There is information about longer duration of maturation of presynaptic elements (Jones 1982).

Distribution of glycoconjugates on the surface of different elements of the nerve cell during its maturation and comparison between these findings and morphological criteria of neuronal maturity allowed us to make some interesting conclusions. It is known that a number of features may serve as criterion for structural maturity of the neuronal cytoplasm, like the development of Nissl substance, cytoskeleton, a decrease of nucleus cytoplasm index. According to our

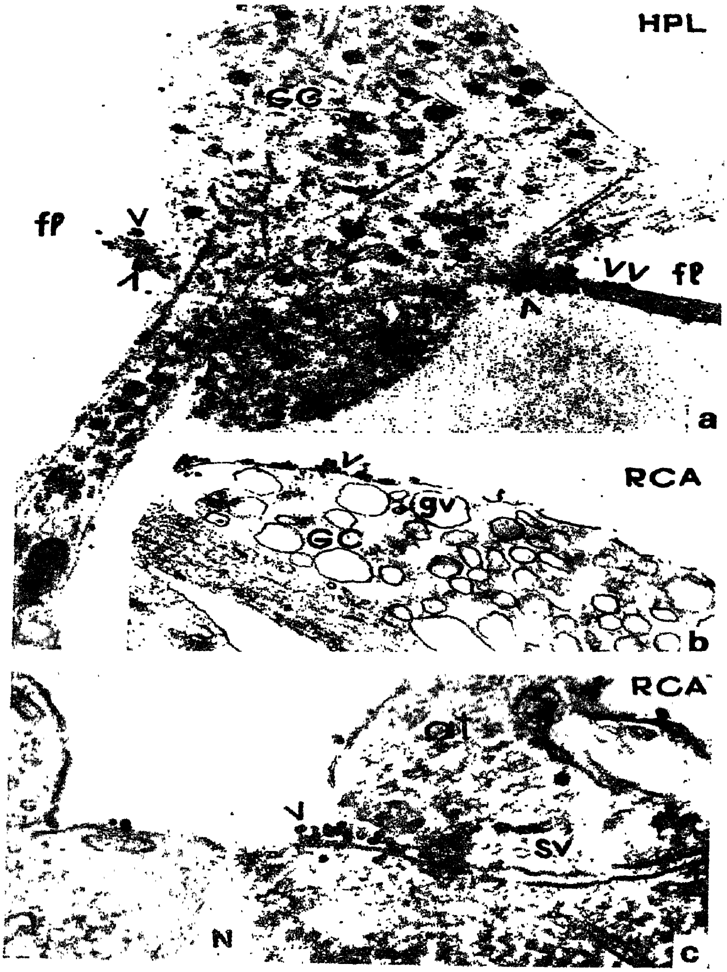


Figure 7 The distribution of glycoconjugates on the surface membrane of the growth cones and synaptic cleft **a**, HPL-Au glycoconjugates on the growth cone (GC) of adrenergic neuron; the label is concentrated mainly on the surface of filopodia **(fl)** (shown by arrows); **b**, RCA-Au glycoconjugates on the distal part of the growth cone (GC) **gv**-growth vesicles; **c**, fragment of axo-somatic contact (the label is displaced to the contact zone): (RCA-Au)

data, the membrane of the spinal cord neuron in the region of axon hillock revealed unitary granules of HPL-conjugates on the 5th day of cultivation: their number increased greatly by the 12th day, though ultrastructural characteristics of cytoplasmic structures did not differ much on the 5th and 12th day of cultivation (figure 8). Thus, it may be suggested that maturation of the membrane can actually be a longer process than that of the intracytoplasmic organelles of the neurons. Eventually only a fullsized maturation of the membrane structural components may accomplish the differentiation of the neuron itself and the formation of specific synaptic connections.

The results of our study on localization of the carbohydrate determinants on different glial cells in monolayer culture suggest that specific molecules fixed on glial surface in a certain sequence may serve the signals providing a directed growth of the axon along the glial cells. Gradients of distribution dentsity of such molecules along the pathways of axonal growth can be one of the most important factors providing a directed axonal growth. In model experiments it was shown that there is no direct transmission of information at far distance in the developing organism. According to mathematical calculations (Crich 1970), some concomitant gradients may act at a distance not exceeding 1-1. 5mm. In connection with these results, our data acquire special importance demonstrating that glial cells of different structures bind lectins in a different way. Binding of HPL-Au and Con A-Au was characteristics of oligodendrocytes, whereas glial cells of astrocytes, both fibrous and protoplasmic, did not possess such ability (figure 9a).

Astrocytes bound greatly LBA-Au, while nerve cells showed unitary granules of LBA-Au conjugates. Fucose determinants revealed with TPL-Au were completely absent on the surface of nerve cells but were seen in considerable amount on the glial cells of all types and their distribution had the same character (figure 9b).

Thus, the differences in distribution of carbohydrate determinants on the surface of nerve and glial cells suggest their participation in certain interactions which provide all stages of cell and tissue neuroontogenesis.

It can be concluded from the above statement that each neuron (a small group of neurons) has a strictly determined topography of distribution of the components not only of the somatic membrane but of the axonal and growth cone membranes. General characteristic of plasmatic membrane is manifested in the topography of glycoconjugates distribution depending on the type of nerve and glial cells which perform synaptic interactions. Thus, each type of neurons and each part of neurons or glial cells has its "signature" on cell surface in the form of different quantity of specific carbohydrate residues which is controlled by cell genome and the mechanism of cell synthesis.

Cytochemical study of glycoconjugates of the surface of nerve and glial cell membranes allowed us to demonstrate the difference not only in the set of the same carbohydrate residues but also in their number. For example, the surface of the neuronal membrane has no L-fucose containing determinants which were the constituents of the surface membrane of glial cells (both oligodendrocytes and astrocytes) (figure 10). The difference in the number of carbohydrate residues was also evident in phenotypically different neurons. The surface membrane of multipolar neurons of the spinal cord was characterized by the presence of multitude of N-acetylglucosamine residues which were unitary on the surface membrane of spinal ganglia neurons. An opposite picture was observed for D-galactose carbohydrate residues. The number of N-acetylgalactosamine residues on the surface membrane of both types of neurons was rather numerous. The residues of D-glucose and D-mannose were unitary on the surface of the neuronal membrane of the spinal cord and spinal ganglia.

It thus turned out that there is a difference in chemical properties of both the phenotypically different neurons and glial cells. It manifests in a different set of glycoconjugates of the cell membrane surface. Such plasmalemmal carbohydrate codes should be involved in intercellular interactions which determine the mechanism of morphogenetic programme both at the level of individual developing neuron and glial cell and the development of the nervous system on the whole.

In this connection the results are of importance showing a heterogenity of distribution of carbohydrate determinants on the surface membrane of different parts of the neuron during its differentiation. Despite the preser-

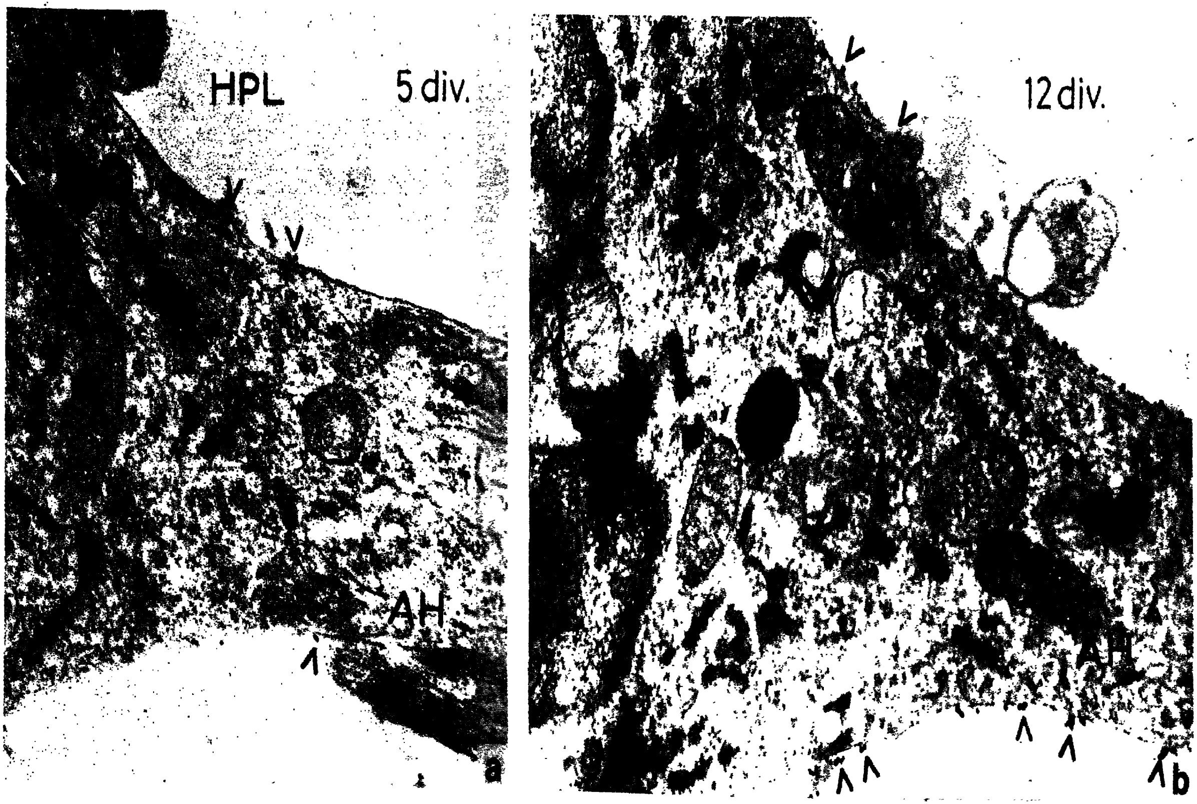


Figure 8 The distribution of HPL-Au glycoconjugates on the surface membrane of the spinal ganglia neuron at different stages of its development

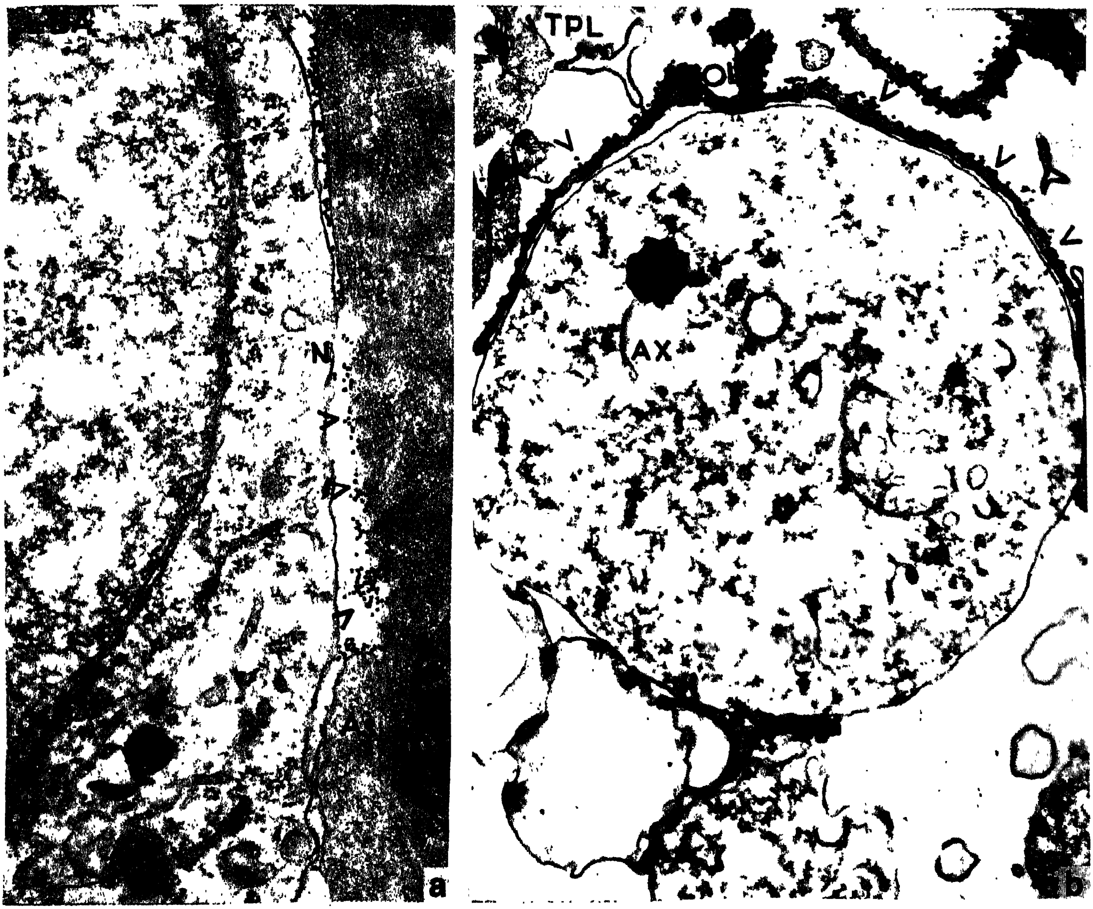


Figure 9 The distribution of LBA-Au (**a**) and TPL-Au (**b**) glycoconjugates on the surface membrane of glial cells. The label is absent on the neuronal and axonal surface membrane: As,astrocyte (**Ol**, oligodendrocyte; **N**-neuron; **Ax**-axon).

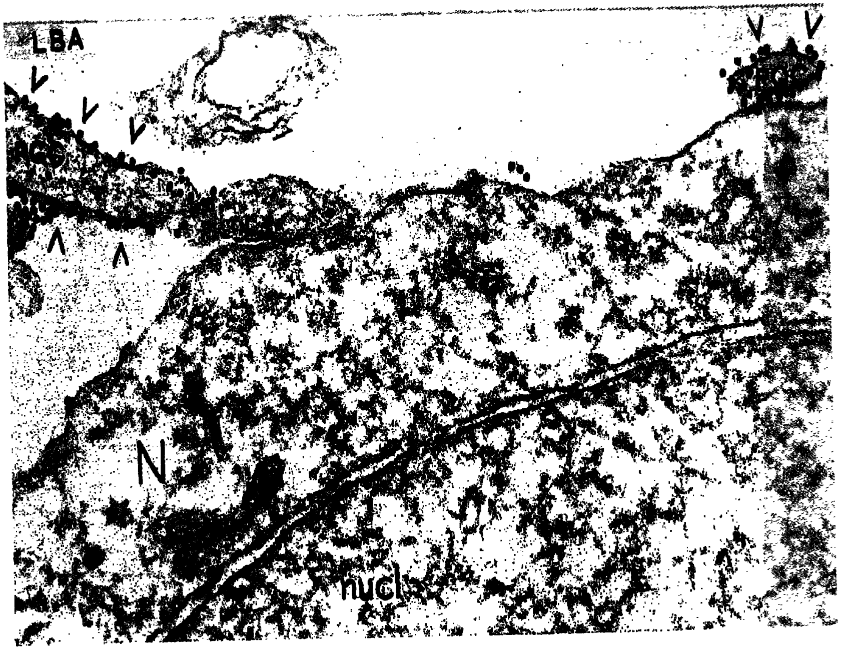


Figure 10 The distribution of LBA-Au glycoconjugates on the surface membrane of the neuron (**N**) and glial cell protrusions (**pgc**), shown by arrows: nucl-nucleus.

vation of general characteristics of carbohydrate code of phenotypically different neurons a change in distribution of surface glycoconjugates takes place as the processes grow. For example, the surface membrane of neuronal growth cones contains D-galactose residues, while other carbohydrate determinants are absent. In the region of synaptic contacts, immediately in the synaptic cleft only N-acetyl-galactosamine residues were observed, and N-acetylglucose determinants were localized in the region restricting the synaptic cleft.

Thus, increasing their surface upon growth and development, the neurons redistribute the membrane glycoconjugates which in all probability, is an essential feature of neuronal differentiation. Obviously, genetic program and the apparatus of the synthesis of the neuron exert a direct effect on the distribution of cell membrane components necessary for the performance of corresponding functions which in turn provide the programme of development of the neurons and intercellular interactions.

References

Crick F Diffusion in embryogenesis; *Nature* **225** 420-422

Foucaud B, Reeb R, Sensenbrenner M and Gombos G 1982 Kinetic and morphological analysis of the preferential adhesion of chick embryoneuronal cells to astroglial cells in culture; *Exp Cell Res* **137** 285-294

Geoghean W D and Aherman G A 1977 Adsorption of horseradish peroxidase ovomucoid and antiimmunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and coat antihuman immunoglobulin G on cell surface at the electron microscopic level: a new method, theory and application; *J Histochem Cytochem* **25** 1187-1200

Grunberger L M and Pfenninger K H 1986 Membrane Glycoproteins of the Nerve Growth Cone: Diversity and Growth Regulation of Oligosaccharides; *J Cell Biol* **103** 1369-1382

Jones D S 1982 Quantitative investigation of development of synaptic junctions in neo cortex; *Neurosci Res Progr Bull* **20** 461-471

Koszinowski U H and Kramer M 1981 Selective inhibition of T suppressor cell function by a monosaccharide; *Nature Lond* **289** 181-184

Mc Laughlin B J and Wood G G 1977 The localization of concanavalin binding sites during photoreceptor synaptogenesis in the chick retina; *Brain Res* **119** 57-71

Nicolson G L 1976 Transmembrane control of the receptors on normal and tumor cells. I. Cytoplasmic influence over cell surface components; *Biochem Biophys Acta* **457** 57-108

Pfenninger K H 1978 Organization of neuronal membranes; *Ann Rev Neurosci* **1** 445-471

—— and Mylie-Pfenninger M F 1979 Properties and dynamics of plasmalemmal glycoconjugates in growing neurities *Progr Brain Res* **51** 83-94

—— and —— 1981 Lectin labeling of sprouting neurons. II. Relative movement and appearance of glycoconjugates during plasmalemmal expansion; *J Cell Biol* **99**,547-559

Readler E, Readler A and Fedhaus S 1981. Varying expressions of lectin receptors within embryonic cell layers of murine cerebral cortex; *Anat Embryol* **162** 21-28

Rostas J A P and Gefferey P L 1981 Maturation of synapses in chiken forebrain; *Neurosci Lett* **25** 299-304

Roth J 1983 Application of lectin-gold complexes for electron microscopic localization of glycoconjugates on thin sections; *J Histochem Cytochem* **31** 547-552

Skibo G G and Koval L M 1984 The ultrastructural features of the synaptogenesis in the monolayer Cultures of the spinal cord; *Neurophysio* **16** 336-343

Sperry R W 1963 Chemoaffinity in the orderly growth of nerve fiber patterns and connections; *Proc Nat Aead Sci USA* **50** 703-710

Wu A M 1987 Lectins: biology, biochemistry, clinical biochemistry; *In. Lectin Proceedings* **6** 723-740 (Berlin, New York: Walter-D-Greyter)

Proc. Indian natn. Sci. Acad. **B56** No. 1 pp. 141-150 (1990)

DNA-Repair in Developing and Aging Brain

KALLURI SUBBA RAO
School of Life Sciences, University of Hyderabad, Hyderabad 500 134

Although much of the existing information regarding DNA repair mechanisms and enzymology has come from prokaryotic systems, particularly *E. coli*, efforts are going on to understand this vital processes in mammalian cells including those in the brain. A few nucleases, both exo and endo in nature, DNA-polymerases, DNA-ligase, and methyl transferase have been identified in the brain. Recent work from this laboratory, as well as from other, revealed the presence of AP-endonucleases in the brain which are responsible for initiating the repair of baseless sites in DNA. Research during the past several years regarding the changes in activity of two repair DNases, DNA-polymerase β, etc. have indicated that there is no deterioration of DNA-repair capacity of the rat cerebral cortex neurons with the advancing age. These results are taken to indicate the inherent plasticity of the central nervous system even during the later stages of life span.

Key Words: DNA repair, Brain, Enzymology

Introduction

Living beings exist in an environment that threatens continuously to damage the genetic material by various events. Damage to DNA can occur through a variety of factors like ultraviolet light, ionizing radiations and scores of chemical substances, both natural and artificially made. Further, DNA can be altered by the very metabolic processes that take place in the cell. Such spontaneous damage can result in loss of bases, modification of bases, strand breaks and also changes in the sequence of bases. On the other hand, it does appear that, for continuous survival and resistance to mutational changes, living organisms have evolved a variety of enzymatic mechanisms for repairing the DNA damage. This article briefly reviews the types of damage that are known to occur in various organisms and also the repair mechanisms that the living systems possess in order to counteract such damage. Then, the information available, including from this laboratory, regarding the DNA-repair processes in brain would be mentioned.

Results and Discussion

In table 1, various events through which cellular DNA can be damaged or altered are summarized. Broadly the events can be classified as those which occur spontaneously as a result of normal metabolism itself including DNA-replication and those events that take place due to a multitude of environmental factors. Spontaneous damage or alterations of DNA include mismatched base pairs, deamination of bases and loss of bases. For example, in a mammalian cell, estimates have been made to show that there is spontaneous loss of purines at a rate of 10,000 per cell generation (Lindahl 1979) Further, similar damage could also occur due to environmental factors which may also result in cross links, strand breaks and formation of bulky adducts.

Table 1 *Various types of DNA damage*

		Causative agents/event
I.	**Spontaneous damage**	
(a)	Mismatched base pairs	DNA-replication/repair
(b)	Alteration in the structure of base like tautomeric shifts, deamination.	Natural metabolism
(c)	Loss of bases (depurination, depyrimidination)	
II.	**Damage due to environmental factors**	
(a)	Baseless sites	Alkylating agents, radiation, free radicals
(b)	Cross links	
(c)	Strand breaks, deletions	
(d)	Alkylation and bulky adducts	
(e)	Oxidative damage	

Table 2 *DNA-Repair mechanisms*

		Enzymes involved
I.	**Reversal of Damage** Photoreactivation, Demethylation, Rejoining of single strand breaks; Direct insertion of lost base.	DNA-Photolyase; O^6-methylguanine—methyl transferase, DNA-ligase; Insertase
II.	**Excision Repair**	
a)	Nucleotide Excision Repair including mismatch repair.	Incision endonuclease, exonuclease, polymerase and ligase
b)	Base Excision Repair	DNA-Glycosylase/AP endonuclease, Excision exonuclease, polymerase and ligase
III.	**Recombinational Repair**	rec ABC, S_1 Nuclease, SSB Protein; Enzymology not clear

Table 2 summarizes different mechanisms and processes evolved by the cell to counteract the DNA damages enumerated in table 1. Table 2 also shows the enzymes known to be involved in the repair processes. From the knowledge available today, the repair events can be broadly divided into two major categories. Those that simply reverse the damage eg., monomerization of pyrimidine dimers by an enzymatic reaction dependent upon light of wave lengths more than 300 nm, removal of methyl groups, simple rejoining of the strand breaks and insertion of the missing base. The other category of DNA repair processes are the nucleotide excision repair and the base excision repair. After initial difference, these two processes merge into a common pathway.

Much of the information about the enzymology of DNA-repair has emanated from prokaryotic systems and in particular the *E. coli.* A number of enzymes involved in various pathways of DNA-repair and mentioned in table 2 have been well studied in *E. coli* but information about similar enzymes from mammalian cells is scanty. It is beyond the scope of this article to describe the detailed properties of these enzymes. However, it is becoming increasingly clear that the nucleotide and base excision repair pathways may be operative in mammalian systems on the lines similar to those observed in *E. coli* although indications are that the process may be much more complex (Cleaver & Karentz 1986). The general steps that constitute the nucleotide and base excision repair in mammalian cells are outlined in figure 1. The first step seems to be the recognition and incision of the damaged site in DNA by an incision endonuclease. From the plethora of endonucleases found in different organisms, it appears these enzymes have rather a broad specificity (Linn 1982). The second step is the excision of the damaged region, which may include some adjacent nucleotides as well by an exonuclease. The third step involves the filling up of the gap by a polymerase using the other strand as template and finally the sealing of the gap by DNA-ligase. In the case of base excision repair, firstly the baseless site formed either spontaneously or by the action of DNA-glycosylase would be recognised by an apurinic/apyrimidinic endonuclease and the site will be incised. From this point the subsequent steps are similar to that of nucleotide excision repair. In figure 1, the left sequence represents nucleotide excision repair while on the right is base excision repair.

Although it is generally believed that the DNA-repair pathways in mammalian and human cells are similar to those observed in prokaryotes, evidence is also gathering to indicate that in these higher systems the

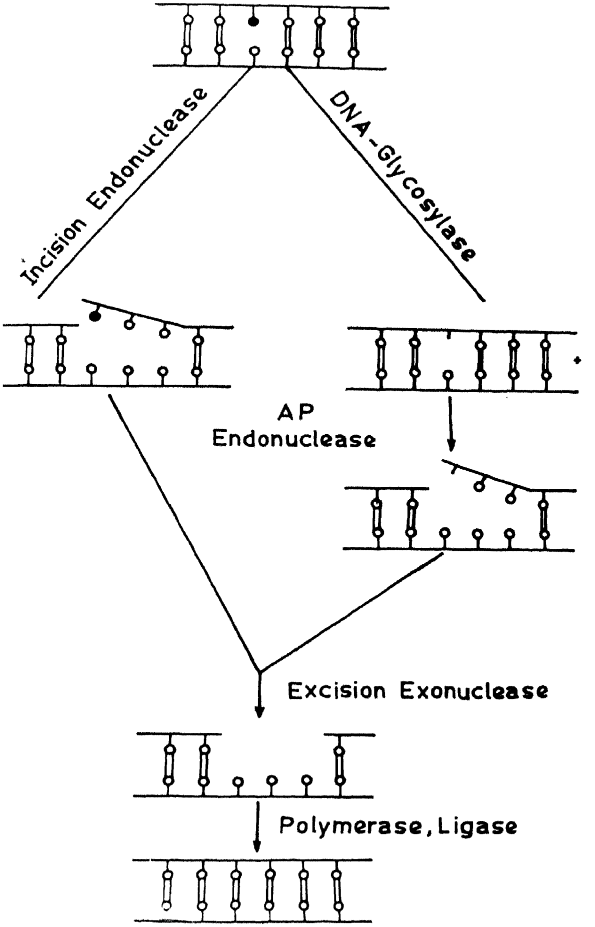


Figure 1 Excision-repair pathways. The sequence of reactions on the left represent nucleotide excision repair pathway while on the right represent base excision repair

mechanism may be more complicated. If one considers only the initial step of the repair of UV induced damage (recognition and incision at pyrimidine dimers) the number of genes involved increase from prokaryotes to man. For example in T4 infected *E. coli*, only a single T4 coded gene is required, the T4 den V gene. However, in *E. coli* itself, four genes, the UVR ABC and D, are required to achieve the same process. In mammalian cells including the human there are indications to suggest that at least 15 gene products are involved in the process. This number could be predicted making use of a series of human diseases characterized by DNA-repair deficiency (e.g. xeroderma pigmentosum) and also a variety of mouse and hamster cell mutants selected *in vitro* (Cleaver 1983, Fischer et al. 1985, Hoy et al. 1985). Efforts are underway in several laboratories to identify and isolate genes responsible for DNA-repair in normal human cell lines as well as from rodent cell lines. The possible overlap of mutations in human and rodent cell lines is also being tested. Already a couple of genes in human cells, involved in UV repair have been cloned and when these genes are introduced into hamster mutant cells, they seem to function well by correcting the defect in UV repair of the mutant cells. Similarly, genes cloned from rodent cells could be tested for correcting DNA-repair deficiency in human cells. Work is continuing in these exiciting lines (Cleaver & Karentz 1986, Thompson 1987, Hoeijmakers 1987).

In spite of the efforts mentioned above, it must be emphasized that enzymological details regarding the DNA-repair reactions in mammalian cells, especially the brain, are scanty.

Brain has two major types of cells, the neurons and glial cells. It is known that in any given species neurons are non dividing in adult brain, and even in glial cells only a small fraction of cells are dividing in adult life and old age (Korr 1980). Thus, it can be considered that most of the cells in an adult brain are postmitotic. However, tremendous metabolic activity is expected to be taking place in neuronal cells in view of the vital functions performed by them. If this were to be so, DNA-repair must assume greater importance in maintaining the normal function of the brain. Since even normal metabolic activity is considered to be sufficient to produce various types of spontaneous DNA-damage, neurons must be equipped with efficient machinery to

Table 3 *DNA-Repair enzyms in E. coli and mammalian/brain cells*

Species	Enzyme	Mode of action
E. coli	1. Incision Endonucleases	Endonuclease activity observed only when all the three gene products are together.
	Uvr ABC gene products	Attacks pyrimidine dimers and bulky adducts. Cleavage on both sides of the damage (Seeberg 1981)
	Endonucleases III	Requires DNA duplex with AP sites as substrate. Associated DNA-Glycosylase activity recognizing 5, 6—saturated thymine photo products
	Endonuclease IV	Requires duplex DNA with AP sites
	Endonuclease V	Requires duplex DNA with AP sites. Also acts on a variety of damaged DNAs
	Endonuclease VI	This is AP endonuclease activity of Exonuclease II.
	Endonuclease VII	Attacks single stranded DNA and polydeoxypyrimidine with depyrimidinated sites. Duplex DNA with AP sites is not a substrate
	2. Excision exonucleases 3′ → 5′ and 5′ → 3′ exonuclease activities	These activities are associated with DNA-polymerase I and III
	exonuclease VII	Attacks single strand DNA or single strand regions of duplex DNA in both 5′ → 3′ and 3′ → 5′ directions.
	rec B C gene products	Act both on single stranded and double stranded DNA in both the directions. Can also excise pyrimidine dimers
	3. Uracil-DNA-glycosylase	Recognises uracil in DNA and cleaves the glycosidic bond to generate free uracil and a baseless site. Several such Glycosylases are known
	4. DNA-polymerase I and III	Template directed addition of deoxynucleotides to 3′-OH end
	5. DNA-Ligase	Rejoining the strand break
	6. Poly (ADPR) synthetase	Transfer of ADP-ribose moieties to form a polymer attached to a macromolecular acceptor (Hayaishi & Ueda 1977)
	7. O^6-methylguanine-DNA-alkyltransferase	Transfer of methyl (or ethyl) group from O^6 position of guanine in DNA to a cysteine residue present in the enzyme itself. Requires duplex DNA
	8. Insertase	Reinsertion of Purine residues into unnicked apurinic sites in DNA.
	9. Photolyase	Light dependent monomerization of pyrimidine dimers without breaking the DNA

Mammalian tissues other than brain	1. Incision Endonucleases	
	Endonuclease of calf thymus and rat liver	Attack uv or x-ray exposed DNA.
	AP-endonuclease of calf thymus and liver, human lymphocytes, skin fibroblasts and placenta	Attack apurinic/apyrimidinic DNA
	2. Excision exonucleases	
	DNase IV of rabbit bone marrow and lung.	Prefers double stranded DNA; attacks from 5′ end; can excise pyrimidine dimers
	'correxonuclease' DNase VIII from human placenta	Attacks single stranded DNA from both directions; can excise pyrimidine dimers
	DNase V from Hela cells	Attacks DNA from both directions
	DNase III from rabbit bone marrow	Attacks both single and double stranded DNA from 3' side.
	DNase VII from human placenta	Attacks both single strand and nicked duplex DNA from 3' side.
	DNA-polymerase β from nuclei of several tissues	Preference for gapped DNA
	DNA-Ligase from a variety of mammalian tissues	Exists in Two forms Ligase I and Ligase II. ATP dependent rejoining of strand breaks.
	Poly (ADP-ribose) synthetase of various cells	Transfer of ADP-ribose moieties to form a polymer attached to a macromolecular acceptor (Hayaishi & Ueda 1977)
	O^6-methylguanine-DNA-methyl transferase from a variety of cells	Transfer of alkyl group from O^6-position of guanine in DNA to cysteine residue present in the enzyme. Requires duplex DNA
	Insertase from human fibroblasts	Insertion of purine residues into unnicked apurinic sites in DNA
	Photolyase of human fibroblasts and leukocytes	Light dependent monomerization of pyrimidine dimers without breaking the phosphodiester bond in DNA
BRAIN	Endonuclease of lamb brain	Attacks single strand DNA releasing oligonucleotides with 5′-phosphate (Healy et al. 1963)
	Acid and alkaline DNases of rat brain	Acid DNase attacks native DNA with pH optimum of 5.0 releasing oligonucleotides with 3′-phosphate; alkaline DNase, pH optimum between 7.4 to 8.9 degrades denatured single stranded DNA releasing 5′-Phosphate oligonucleotides (Sung 1968)
	Acid DNase (UV DNase) and alkaline DNase (AP DNase) from chick and rat brain	Acid DNase (UV DNase) attacks native DNA or UV irradiated native DNA; alkaline DNAse (AP DNase) acts on either denatured DNA or depurinated DNA (for details, see figure 2 and text)
	AP-endodeoxynuclease from rat neocortex chromatin	Incises near AP sites of a supercoiled DNA. Optimal *p*H, 7.8 Requires Mg^{++} or Mn^{++} (Ivanov et al. 1988).
	Exonuclease (DNase B III) from rat brain neuronal nuclei	Excises single strand DNA of single strand termini in a duplex DNA in 5′ → 3′ direction.

	Nucleoside-5′-monophosphate is released (Ivanov et al. 1983).
Uracil-N-Glycosylase of human foetal brain	Action similar to that found in other mammalian tissues (Krokan et al. 1983).
DNA-Polymerase β of rat and mouse neurons	Action same as in other tissues (Hubscher et al. 1979).
DNA-Ligase of neuronal and glial cells of guinea pig brain and rat cerebellum.	Action same as in *E. coli* but requires ATP. (Inoue & Kato 1980)
Poly (ADPR) synthase of bovine brain.	Action same as in other mammalian tissues (Bilen et al. 1981
O^6-alkyl-Guanine-DNA-alkyl transferase of rat and human brain.	Action same as in other mammalian tissues (Wiestler 1984)
Photolyase of Marsupial brain	Monomerization of Pyrimidine dimers (Rupert 1985)

Most of the information summarized in this table is obtained from Friedberg 1985 and Linn 1982. Where the specific information is taken from a different source, the reference is cited

repair such damage without involving the process of replication. Otherwise, neurons would be facing a serious threat of accumulaion of DNA-damage which might subsequently reflect in transcriptional and translational errors which should be detrimental not only for the neurons themselves but to the whole organism as well. It is in this context that a search has begun for the DNA repair capacity of the brain and indeed some DNA-repair enzymes have been detected in brain (Table 3) although details of their mechanism of action is still awaited. For the sake of comparison, those DNA-repair enzymes detected in *E. coli* and mammalian tissues other than brain are also listed in Table 3. Probably the first demonstration of the presence of two brain DNases, the acid and alkaline, has come from the laboratory of Sung (1968). One of them acts optimally at *p*H 5.0 hydrolyzing preferentially native DNA, while the other one showing an optimal *p*H between 7.4 and 8.9, acts preferentially on heat denatured DNA. Both these enzymes appeared to be localized in the mitochondrial/microsomal fraction and are endonucleases. The alkaline DNase was thought to be similar to a phosphodiesterase isolated earlier from lamb (Healy et al. 1963). However, the possible role of these DNases remained obscure. The presence of an acid DNase in. human cerebrum and cerebellum was

Table 4 *Acid DNAse activity in foetal human brain*

Gestational Age	Cerebrum	Cerebellum
9 – 20 wk	6.4 ± 1.08 (8)	
24 – 32 wk	4.6 ± 0.57 (3)	5.9 ± 1.27 (3)
Full term	3.4 ± 0.88 (6)	10.1 ± 3.12 (6)

Activity expressed as μg of acid soluble deoxyribose-P released/mg protein/2 hr. In parentheses, number of analyses. Data recalculated from Subba Rao (1973)

Table 5 *DNA content and DNase activities in developing and old chick brain*

Age (days)	DNA content (μ/g tissue wt)	Acid DNase	Alkaline DNase
16 – (E)	600	14.5	18.8
1 – (PH)	800	11.0	21.3
10 – (PH)	900	8.5	19.4
730 – (PH)	1500	2.0	9.4

Enzyme activities expressed as in table 4. E, embryonic life; pH, post-hatching. Values are averages of 5 to 10 experiments. Data recalculated from Shrivastaw and Subba Rao (1975) demonstrated by Subba Rao (1973). Highest activity of this enzyme was noticed during the early stages of embryonic development. With the advancement of gestation, the activity decreased. In cerebellum, however, the activity increased with gestation upto full term (Table 4). This temporal pattern of activity correlated well with the rates of DNA-synthesis in these regions of developing human foetal brain (Dobbing 1971).

These observations from human brain prompted us to continue our investigations in a systematic manner using other easily obtainable species. Both in chick brain (Table 5) and in rat brain we have been able to identify two major DNases comparable to the acid and alkaline DNases described by Sung (1968) earlier. Both acid and alkaline DNases exhibited maximum activity during embryonic life, at the time of rapid cellular proliferation. With the advancement of age the acid DNase showed a marked decline and this low activity remains through rest of life. On the other hand, alkaline DNase continued to show high activity through out the life span. The one showing acidic *p*H optimum has been purified to homogeneity in our laboratory. The other enzyme, the so called alkaline DNase, has been purified to near homogeneity and some of the properties of both these enzymes have been studied and are summarized in figure 2. As can be seen the so called acid DNase shows preference towards native DNA and UV irradiated DNA and does not show any activity towards either single stranded DNA or depurinated DNA. In view of this substrate preference this enzyme is renamed by us as UV DNase. In contrast, the DNase with alkaline pH optimum attacks only single stranded DNA or depurinated DNA. To indicate this property, this enzyme is now referred by us as AP DNase. Both these enzymes are found to be endonucleases and are located mainly in the mitochondrial/lysosomal fraction but are also present to a significant extent in nuclear fraction.

Since there is a good possibility that the two enzymes under study may be involved in DNA replication/repair in brain, a systematic study to measure these activities, along with DNA-polymerase β, considered to be a repair enzyme and found to be present in the neurons (Waser et al. 1979) at different ages of the rat has been carried out. The DNA content of the tissue was also measured. The results are depicted in figure 3. It is clear that the specific activities of both the DNases were high

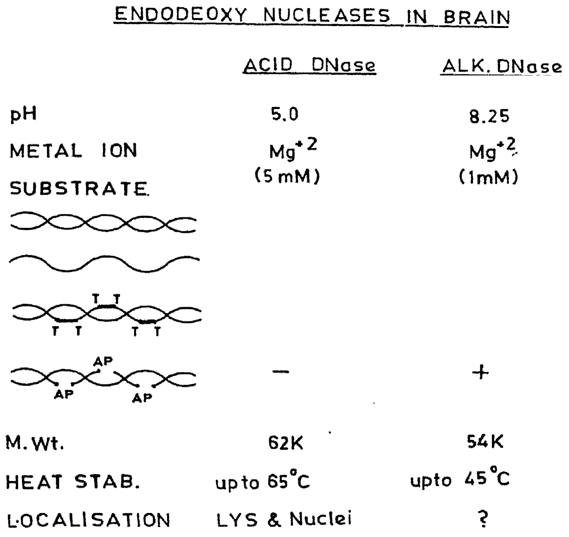


Figure 2 Endoexyribonucleases of rat brain

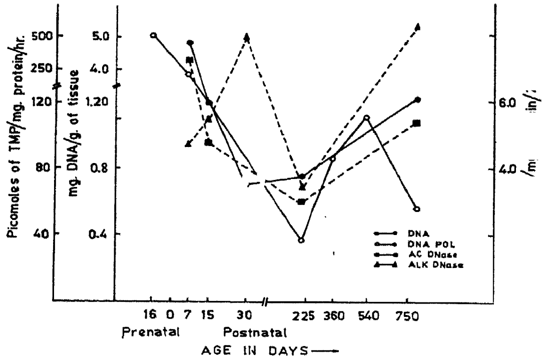


Figure 3 DNA content, DNA-polymerase β and DNase activities in white matter of rat brain at different ages. Data taken from Subba Rao Subba Rao, (1982) and Subba Rao and Subba Rao, (1984)

during the early stages of development. A marked decline in the activity of acid DNase (UV DNase) occurred during adult hood. However, the activity increased again between 225 and 750 days in concomitance to the DNA increase and to a second peak of DNA polymerase activity. In contrast, alkaline DNase (AP DNase) exhibited an initial, reasonably high specific activity and continued to increase with age up to 60 days. The activity decreased at 225 days but increased again to a maximum level by 750 days (Subba Rao & Subba Rao 1982, 1984).

These results along with our earlier results in human and chick brain (Subba Rao 1973, Shrivastav & Subba Rao 1975) are taken to indicate that the so called acid DNase may be involved in a process that is related to cell proliferation i.e., DNA-replication or DNA-repair that is associated with DNA replication, while the alkaline DNase may be a DNA-repair enzyme. It is interesting that this activity is very high in aged brain pointing out efficient DNA-repair activity even in aging brain. This contention is supported by the second peak of DNA-polymerase β activity around 1½ years of age. In this connection, it must be mentioned that recently Ivanov et al. (1988) have demonstrated the presence of a AP-endodeoxyribonuclease in rat neocortex chromatin and they have also presented evidence for a pathway in rat brain for DNA-repair wherein this AP nuclease with the help of an exonuclease and DNA-polymerase β can repair an AP site in DNA completely.

The above results of significantly high activity of DNA-repair enzymes even in old brain could probably be taken to indicate neural plasticity in order to counteract the accumulated DNA-damage over the period. To check the DNA-repair capacity of neuronal cells at different ages, we have performed experiments in a different direction. Neuronal cells were isolated by the procedures established in our laboratory (Usha Rani et al. 1983) and the cells so isolated were incubated with [^{3}H]—thymidine in the presence or absence of hydroxyurea (HU) and the incorporation into DNA was followed. Table 6 shows the results obtained. It is clear that neurons both from young and adult ages incorporate [^{3}H]—thymidine into DNA quite efficiently although the adult neurons incorporated only one tenth of the label as compared to the young. Furthermore, while HU inhibited the incorporation by about 77% in young, no such effect could be seen in adult neurons indicating that replicative DNA-synthesis is virtually absent in neurons from adult rat brain. Implicit in these results is also the demonstration that the incorporation of [^{3}H]—thymidine into DNA of adult neuronal cells can safely be taken as a measure of DNA-repair synthesis (unscheduled DNA synthesis). It therefore appears that neuronal cells from adult brain offer a good model system to study DNA-repair uncomplicated with the possibility of simultaneous DNA-replication. It is interesting that in 1 day old cerebral neurons, HU was able to inhibit the thymidine incorporation markedly. This may be an indication of the residual replicative activity in these cells. Also, part of this inhibition could be due to the ability of HU to inhibit thymidine kinase activity at this age as was demonstrated by us recently (Prabhakar & Subba Rao 1985). When the neuronal cells isolated from young and adult rat brain were first challenged with UV light at 2 doses and then incubated with [^{3}H]—thymidine in the presence of HU, the mutagenic challenge resulted in an increased incorporation of the label into DNA of young as well as adult neurons which can be taken as increased unscheduled DNA synthesis as a response to the damage caused by UV light (Table 7). If one looks at the increase of incorporation in terms of percentage of the control, it could be seen that the adult neurons show a 100% increase while the young about 50%. However, increasing the UV dose had not resulted in any further increase in DNA-repair synthesis. These data, although quite preliminary and require to be confirmed, are taken to suggest that

Table 6 *Incorporation of [^{3}H]—thymidine* in vitro *into D/DNA of neuronal cells isolated from young and adult rat brain: the effect of hydroxyurea (HU)*

Age	DPM/mg of DNA		%
	Control	+ HU (5 mM)	inhibition
Young (1 day old)	292,000	65,590	77.5
Adult (6 Mo)	23,500	22,000	6.3

Neuronal cells ($2{\cdot}4\times10^6$ cells/ml) were suspended in Minimum essential medium with 10% foetal bovine serum and 10 μCi/ml. [^{3}H]—thymidine. Incubation was for 2 hr

Table 7 *Effect of UV irradiation (254 nm) on the DNA-repair synthesis in young and adult rat brain neurons*

Age	DPM/mg of DNA		
	Control	20 J/m^2	40 J/m^2
Young	65,590	91,220	87,320
Adult	22,000	51,080	34,100

For details, please see table 6

adult neurons possess DNA-repair machinery which seems to be as efficient as that in young.

It is clear from the literature that most of the information about DNA-repair mechanisms has come from prokaryotic systems especially the *E. coli*. In *E. coli* systems, majority of the genes responsible have been identified and mapped. DNA damage can occur through a variety of agents and sometimes it is possible that a given agent can cause damage in more than one way. Thus simple exposure to UV light may induce predominantly pyrimidine dimers but strand breaks can also occur under the conditions. Similarly alkylating agents like methylmethanesulfonate can modify the bases through methylation but this may also lead to the loss of such bases resulting in the formation of baseless sites. Further, it is also not clear as to which type of DNA-repair pathway would come into operation against different types of damage. Since much of the DNA-repair genetics in *E. coli* has been worked out, it should be possible to gain precise information about the specificities between the type of DNA-damage and the mode of DNA-repair.

The number of DNA repair enzymes discovered so far in mammalian cells is less as compared to that from *E. coli* (Table 3). It is certain that many more proteins are to be discovered in mammalian tissues in view of the indication already obtained that for any given event of DNA-repair, there may be many more genes involved in mammalian systems. A major obstacle for study of DNA-repair in mammalian cells is the lack of sufficient details about the mechanism of action of even those enzymes/proteins already identified. This is mainly because of the lack of mutants unlike in the case of *E. coli*. It is fortuitous that cell lines from patients of diseases like xeroderma pigmentosum and some mutant rodent cell lines (Cleaver & Karentz 1986) have helped a great deal in obtaining whatever information is available today. Since successful cloning of some human DNA-repair genes and also of some rodent genes is reported (Cleaver & Karentz 1986), it can be expected that valuable information regarding DNA-repair in higher systems would be available soon.

It may be necessary to examine the DNA-repair process separately in post-mitotic cells like neurons. The manner in which such cells would handle a given type of damage should be a subject of worthwhile study. Our preliminary studies point out that whatever neurons that are surviving in old age seem to possess good capacity to achieve DNA-repair. However this needs to be studied in further detail.

References

Bilen J, Ittel M E, Nidergang C, Okazaki H and Mandel P 1981 Poly (Adenosine diphosphosphate ribose) polymerase activity in neuronal and glial nuclei from bovine cerebrum; *Neurochem. Res.* **6** 1253-1263

Cleaver J E 1983 *Xeroderma pigmentosum;* in *The Metabolic Basis of Inherited Disease* (5th edn.) pp. 1227-1248 eds J B Stanbury, J B Wyngaarden, D S Frederickson, J L Goldstein and M S Brown (New York: McGraw-Hill)

——, and Karentz D 1986 DNA-repair in man: Regulation by a multigene family and association with human disease; *Bioassays* **6** 122-127

Dobbing J 1971 Undernutrition and the developing brain: The use of animal models to elucidate the human problem: in *Chemistry and Brain Development* pp 319-412 eds R Paoletti and A N Davidson (New York: Plenum Press)

Fischer E, Keijzer W, Thielmann H W, Popando O, Bohnert E, Edler L, Jung E G and Bootsma D 1985 A ninth complementation group in *Xeroderma pigmentosum XPL; Mutt. Res.* **145** 217-225

Friedberg E C 1985 in *DNA-Repair ed.* E C Friedberg (New York: W H Freeman)

Hayaishi O and Ueda K 1977 (Poly ADP-ribose) and ADP-ribosylation of proteins; *Ann. Rev. Biochem.* **46** 95-116

Healy J W, Stollar D, Simon M I and Levine L 1963 Characterization of phosphodiesterase from lamb brain; *Arch. Biochem. Biophys.* **103** 461-468

Hoeijmakers Jan H J 1987 Characterization of genes and proteins involved in excision repair of human cells; *J. Cell. Sci.* (suppl.) **6** 111-125

Hoy C A, Thompson L H, Mooney C L and Salazar E P 1985 Defective DNA cross-link removal in Chinese hamster cell mutants hypersensitive to bifunctional alkylating agents; *Cancer Res.* **45** 1737-1743

Hubscher U, Kuenzle C C and Spadari S 1979 Functional roles of DNA polymerase β and γ: *Proc. Natl. Acad. Sci,* USA **76** 2316 2320

noue I and Kato T 1980 Nuclear DNA-ligase and its action on chromation DNA in Neuronal, Glial and Liver nuclei isolated from adult guinea pigs; *J. Neurochem.* **34** 1574-1583

Ivanov V A, Gaziev A I and Tretyak T M 1983 Exodeoxyribonuclease from rat brain specific for single-stranded DNA; *Eur. J. Biochem.* **137** 517-522

——, Tretyak T M and Afonin Y N 1988 Excision of apurinic and/or apyrimidinic sites from DNA by nucleolytical enzymes from rat brain; *Eur. J. Biochem.* **172** 155-159

Korr H 1980 in *Proliferation of Different Cell Types in the Brain.* ed. Hubert Korr (Berlin: Springer Verlag)

Krokan H, Haugen A, Myrnes B and Guddal P H 1983 Repair of premutagenic DNA lesions in human foetal tissues: Evidence for low levels of 0^6-methylguanine-DNA methyl transferase and uracil DNA glycosylase activity in some tissues. *Carcinogenesis* 4 1559-1564

Lindahl T 1979 DNA-glycosylases, endonucleases for apurinic/apyrimidinic sites and base excision repair. *Prog. Nucleic Acid Res. Mol. Biol.* **22** 135-192

Linn S 1982 Nucleases involved in DNA-repair; in *Nucleases* pp 59-83 eds S M Linn and R J Roberts: (New York: Cold Spring Harbor Laboratory Publication)

Prabhakar V and Subba Rao K 1985 Hydroxyurea inhibition of thymidine kinase in proliferating and non-proliferatiing cells of rat brain; *J. Biosci.* **7** 1-6

Rupert C S 1975 Enzymatic photoreactiviation: Overview; in *Molecular Mechanisms for DNA-Repair of DNA.* Part A pp 73-87 eds P C Hanawalt and R B Setlow (New York: Plenum)

Seeberg E 1981 Multiprotein interactions in strand cleavage of DNA damaged by UV and chemicals; *Proc. Nucleic Acid. Res. Mol. Biol.* **26** 217-226

Shrivastaw K P and Subba Rao K 1975 Studies on the levels of DNA, RNA, protein and DNases in developing and old chick brain. *J. Neurochem.* **25** 861-865

Subba Rao K 1973 Acid deoxyribonuclease activity in developing human foetal brain; *Life.* **12** 89-96

Subba Rao K V and Subba Rao K 1982 Changes in DNA, RNA, protein and the activities of acid and alkaline DNases in grey and white matter regions of developing and aging rat cerebrum. *Mech. Age. Develop* **18.** 225-238

—— and ---- 1984 Increased DNA-polymerase β activity in different regions of aging rat brain. *Biochem. Intnl.* **9** 391-397

Sung S, C 1968 Deoxyribonucleases from rat brain. *J. Neurochem.* **15** 477-481

Thompson L H 1987 Dissecting human DNA-repair; *Photodermatology* **4** 63-65

Usha Rani B, Singh N I, Ray A and Subba Rao K 1983 Isolation procedure for neuronal and astroglial cell enriched fractions from chick brain of different ages; *J. Neurosci. Res.* **10** 101-105

Waser J, Hubscher U, Kuenzle C C and Spadari S 1979 DNA polymerase β from brain neurons is a repair enzyme. *Eur. J. Biochem.* **97** 361-368

Wiestler O, Kleihues P and Pegg A E 1984 O^6-Alkylguanine-DNA alkyltransferase activity in human brain and brain tumors; *Carcinogenesis* **5** 121-124

Development Changes in Auditory Brainstem Response (ABR): Effect of Some Risk Factors During Infancy

O P TANDON

Department of Physiology, University College of Medical Sciences & G T B Hospital, Delhi, India

ABR of premature infants showed the following differences as compared to the full term new borns or adults:

(i) The amplitude of response particularly of wave V was smaller.
(ii) The Latencies of all waves were less.
(iii) The Change in latency with increasing stimulus was more marked.
(iv) The absolute latency of wave V showed greater change with age than that of wave I.

The adult pattern of ABR developed within 2 years of birth. The different components of the response matured differently. The effect of malnutrition on the development of ABR was studied. Besides absolute latency and interpeak latency (IPL), there was many morphological changes seen in such infants. The critical period during which malnutrition affected ABR was in the early postnatal life.

Molecular Mechanisms of Excitability in Development

N S VESELOVSKY, P G KOSTYUK and S A FEDULOVA

A A Bogomoletz Institute of Physiology, Academy of Sciences of the Ukrainian SSR, Kiev, USSR

Distribution of different types of ionic channels carrying inward currents was studied in the somatic membrane of rat dorsal root ganglion neurons in three age groups: 5-9 days, 45 days and 90 days postnatally, The number of neurons whose membrane contained simultaneously four types of inward current channels ("fast" tetrodotoxin-sensitive and "slow" tetrodotoxin-resistant sodium, low-and high-threshold calcium one's) was progressively reduced in successive groups. These changes were due to disappearance of "slow" sodium and low-threshold calcium channels from the membrane. The number of neurons whose somatic membrane contained only two types of inward current channels ("fast" sodium and high-threshold calcium) had increased, respectively. Correlations between densities of various types of inward currents were studied. A linear relationship was found in neurons with "slow" sodium current between the densities of this and high-threshold calcium currents. Inverse correlation was observed between the densities of "fast" sodium and high-threshold calcium currents in cells in which somatic membrane had only these two current components. In all cells studied a "wash-out" of high-threshold calcium current was observed during intracellular dialysis with saline solutions. The effect of recovery in calcium conductivity produced by intracellular application of the cAMP-ATP-Mg^{2+} complex was different in neurons with different inward current combinations.

Molecular Basis of Hormone Dependent Brain Development

N KOCHUPILLAI

Department of Endocrinology & Metabolism, All India Institute of Medical Sciences, New Delhi, India

Over the years, an important paradigm for the study of hormones and brain development has been thyroxine dependent growth and differentiation of the brain of a variety of species including man. Till recently the parameters adopted for such studies have been morphological, neurochemical, histochemical and electrophysiological. In the light of recent insights into the molecular biology of hormone dependent tissue differentiation, a variety of conceptual and technical advancements are in the making. Questions related to thyroxine dependent brain development which are of crucial importance from the point of view of developing countries with significant burden of nutritional iodine deficiency, can now be addressed in modern molecular biological terms.

Quantitative Golgi Study of a Kitten Brain Stem and Spinal Neurons After Central or Peripheral Deafferentation

K V SHULEIKINA, N G GLADKOVICH and A D VOROBJEVA

Institute of Higher Nervous Activity & Neurophysiology, Moscow, USSR

The purpose of the work was to compare the dynamic changes in the developing nerve cells geometry after peripheral or central deafferentation.

Neurons of the sensory trigeminal nuclei and the neighbouring reticular formation were studied after partial peripheral deafferentation caused by section of the nerve. Lingualis. The spinal cervical neurons of VII, VIII and IX laminae were studied after partial central deafferentation caused by destruction of the medullary nucleus reticularis magnocellularis. Kittens were operated on day 7. The Golgi sections of the brain stem in 30-45-day-old intact or operated kittens were studied with the aid of a computer morphometry applied differentially to various previously defined cell types.

Most basic and diverse changes were observed in the sensory trigeminal neurons after the lingual nerve section. The most dramatic destructive changes were shown by bushy neurons. This is probably due to the loss of dominant afferent axons. Polyconvergent arborescent neurons revealed clearly marked constructive changes that may reflect the compensatory processes of searching for contacts with new afferent terminals. The neurons of reticular formation after the lingual nerve section demonstrated a slight structual reorganization. Lateral reticular neurons lying near the border of N.V. showed the changes in a large number of parameters analyzed. It may be assumed that the partial loss of peripheral inflow to the non-sensory and non-relay neurons did not cause a significant structural reorganization. The changes of the geometry of spinal neurons after partial reticulo-spinal deafferentation were more marked than in the reticular neurons, but were less significant than in trigeminal ones. They consisted in the increase of cell size, total length of dendrites and reorientation of the dendrites from lateral (more damaged input) to medial (less damaged input) direction. Perhaps, at the earlier stages of development the reticulo-spinal deafferentation may cause a more significant structural reorganization.

It has been found that the ENT transplantation was not followed by improvement of the PAB while there was some improvement of CB behavior. It was not obvious in all cases. The behaviour changes were dependent upon the state of transplant.

It is suggested that the "power" of the transplant which was introduced only in one cavity is not enough for the recovery of behaviour. It is also possible that the ENT transplantation has some intrinsic limitations for providing functional recovery. Probably the results were dependent upon the method of conditioning employed in this research.

LIST OF PARTICIPANTS

1. Dr K N AGARWAL
   Department of Paediatrics
   Banaras Hindu University
   Varanasi-221 005

2. Professor B K BACHHAWAT
   Department of Biochemistry
   University of Delhi (South Campus)
   Benito Juarex Road
   New Delhi-110 021

3. Dr RANJEETA BANERJEE
   Department of Anatomy
   All India Instittute of Medical Sciences
   New Delhi-110 029

4. Professor V BIJLANI
   Department of Anatomy
   All India Institute of Medical Sciences
   New Delhi-110 029

5. Dr T DESIRAJU
   Department of Neurophysiology
   National Institute of Mental Health &
   Neurosciences
   Bangalore-500 029

6. Professor M GOURIE DEVI
   Professor of Neurology
   National Institute of Mental Health and
   Neurosciences
   Bangalore-560 029

7. Dr B N DHAWAN
   Director
   Central Drug Research Institute
   PB No. 173
   Lucknow-226 001

8. Dr GOMATTHY GOPINATH
   Department of Anatomy
   All India Institute of Medical Sciences
   New Delhi-110 029

9. Dr ARCHANA HARYAN
   Department of Anatomy
   All India Institute of Medical Sciences
   New Delhi-110 029.

10. Professor O P JAGGI
    Patel Chest Institute
    Delhi

11. Dr DEEPAK N JOSHI
    Department of Neurosurgery
    B J Medical College & Civil Hospital
    Ahmedabad-380 016

12. Dr N KOCHUPILLAI
    Department of Medicine
    All India Institute of Medical Sciences
    New Delhi-110 029

13. Miss FARZANA MASOOD
    Department of Anatomy
    All India Institute of Medical Sciences
    New Delhi-110 029

14. Dr RAJ D MEHRA
    Department of Anatomy
    All India Institute of Medical Sciences
    New Delhi-110 029

15. Dr K P MISHRA
    Department of Paediatrics
    Institute of Medical Sciences
    Banaras Hindu University
    Varanasi-221 005

16. Dr V MOHAN KUMAR
    Department of Neurosurgerv
    All India Institute of Medical Sciences
    New Delhi-110 029

17. Dr S MOHNATY
    Department of Neurosurgery
    Institute of Medical Sciences
    Banaras Hindu University
    Varanasi-221 005

18. Dr K DILIP MURTHY
    Department of Neurophysiology
    National Institute of Mental Health and
    Neurosciences
    Bangalore-560 029

19. Dr R MUTHUSAMY
Reader & Head
Department of Anatomy
Dr A L Mudaliar Postgraduate Institute of Basic Medical Sciences
University of Madras
Madras-600 113

20. Professor A S PAINTAL
Director-General
Indian Council of Medical Research
Ansari Nagar
New Delhi-110 029

21. Dr REBECCA PRAPURNA
School of Life Sciences
University of Hyderabad
Hyderabad-500 134

22. Dr B RAMAMURTHY
Department of Neurosurgery
Dr Achanta Lakshmipathi Neurosurgical Centre
Voluntary Health Services
Adyar
Madras-600 020

23. Professor VINKOBA RAO
Officer-in-charge
Madurai Medical College
Madurai

24. Dr TARA SANKAR ROY
Department of Anatomy
All India Institute of Medical Sciences
New Delhi-110 029

25. Professor SUBIMAL ROY
Department of Pathology
All India Institute of Medical Sciences
New Delhi-110 029

26. Dr CHITRA SARKAR
Department of Pathology
All India Institute of Medical Sciences
New Delhi-110 029

27. Professor P S SASTRY
Professor of Biochemistry
Indian Institute of Science
Bangalore-560 012

28. Professor G C SEN SHARMA
Department of Anatomy
Institute of Medical Sciences
Banaras Hindu University
Varanasi-221 005

29. Dr ASHOK KUMAR SHETTY
Department of Anatomy
All India Institute of Medical Sciences
New Delhi-110 029

30. Dr K SUBHA RAO
School of Life Sciences
University of Hyderabad
Hyderabad

31. Dr O P TANDON
Department of Physiology
University College of Medical Sciences & GTB Hospital
Delhi

32. Professor P N TANDON
Department of Neurosurgery
All India Institute of Medical Sciences
New Delhi-110 029

33. Professor N H WADIA
Director of Neurology
Jaslok Hospital & Research Centre and
Consultant Neurologist
J J Group of Hospital & Grant Medical College
Bombay

34. Dr SHASHI WADHWA
Department of Anatomy
All India Institute of Medical Sciences
New Delhi-110 029